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Some Effects of Diuron-Dbsa on Early Growth, Development and Certain Respiratory Enzymes of Soybean.

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SOME EFFECTS OF DIURON-DBSA ON EARLY GROWTH, DEVELOPMENT AND CERTAIN RESPIRATORY ENZYMES OF SOYBEAN

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ABSTRACT

Soybeans (Glycine max L.) have become increasingly important as a crop, and interest in chemical weed control for this crop has grown. In addition, the need for more information concerning the effects of herbicides on soybeans and other crops as well as on weed species has been recognized. Various effects of DIURON-DBSA on young soybeans were studied in this research.

In most of these studies, Lee soybeans were planted in flats of a sandy loam soil in the greenhouse. Plants were treated at rates of zero and three-eighths of a pound per acre of DIURON-DBSA on the eighth day. The flats were moved to a growth chamber with a temperature of 85 degrees Fahrenheit and a light intensity of approximately 500 foot-candles. The various effects of the herbicide treatment were usually determined at one, four, and seven days after herbicide treatment.

Herbicide injury was restricted to the sites of application when the shoots were sprayed or dipped in the herbicide solution. The herbicide was absorbed by the roots and produced uniform top kill when applied in the nutrient solution at concentrations exceeding 0.1 ppm.

The herbicide treatment reduced the height growth of stems, the greatest reduction occurring in the internode between the primary leaves and the first trifoliate leaf. Fresh weights of primary leaves were less in the herbicide treated plants.

The total amino nitrogen content per primary leaf was unchanged by the herbicide treatment. However, the portion that was found in the 1,000 X G supernatants was increased by 33 per cent by the herbicide treatment.

Microscopic examination of anatomical slides showed that the roots and the first internode were unaffected by the herbicide treatment. Cell sizes were generally less in the primary leaves. Enlargement and differentiation of xylem vessels were particularly inhibited.

Dark treatment prior to herbicide treatment hastened the appearance of injury symptoms in primary leaves. Respiratory activity was reduced by the herbicide treatment but tended to recover when the rate of treatment was one-fifth pound per acre of DIURON-DBSA or less. Dark treatment prior to the herbicide treatment reduced respiratory activity over that of plants receiving a light pretreatment, even though both received light after the herbicide treatment. With light before herbicide treatment, respiratory activity returned to the level of untreated plants four days after herbicide treatment. Respiratory quotients increased sharply with dark treatment after the herbicide treatment.

Cytochrome oxidase activity could not be demonstrated in "particles" from green stems. Active oxidation of succinate, malate, and pyruvate was found for "particles" from etiolated cotyledons. The herbicide appeared to reduce this enzymatic activity.

The cell wall fraction from primary leaves was found to have high ascorbic acid oxidase activity. Less activity was found in the cotyledons. However, the herbicide treatment had little effect on enzymatic activity in either case.

Polyphenol oxidase activity was low, but the herbicide treatment reduced this activity by the seventh day after the herbicide treatment.

Catalase activity was high and increased with leaf age. The herbicide treatment reduced catalase activity by approximately one-half.

Peroxidase activity was low, although enzymatic activity increased with leaf age. Peroxidase activity was reduced by the herbicide treatment in a similar manner to catalase activity.

Glycolic acid oxidase activity was comparable to ascorbic acid oxidase activity. Acetaldehyde bisulfite and sodium bisulfite gave only partial inhibition of this activity. Glycolic acid oxidase activity increased with primary leaf age. The effect of the herbicide treatment was to reduce enzyme activity by the seventh day.

INTRODUCTION

Soybeans have become an increasingly important crop in the United States in recent years. The beginning of World War II eliminated Asiatic sources of soybeans and soybean products, which encouraged the domestic production of this crop. Soybean yields have been increased through the development of new varieties and improved cultural practices. Responses to chemical weed control practices have not been spectacular although interest in this phase of soybean culture has grown (Howell, 1960).

Soybeans are often grown in rotation with corn, cotton and other crops. Chemical weed control in these crops has become common, and there is interest in the residual effects of herbicides on succeeding crops such as soybeans. Diuron [3-(3,4-dichlorophenyl)-1,1-dimethyl urea] is a commonly used herbicide for the control of weeds in cotton fields and could have adverse effects on soybean crops during the following season. Other than the possible gross injurious effects of herbicides, there is considerable interest in the mechanism of action of herbicides or the mechanism of resistance of tolerant plants.

In this study, a formulation of diuron and a detergent, dodecyl benzene sulfonate, was used. Both the gross anatomical and microscopic effects as well as certain physiological effects were studied.

LITERATURE REVIEW

Anatomy and Physiology of the Soybean

The soybean (Glycine max L. Merrill) is a legume of great genetic and morphological diversity. It is a summer annual requiring 75-200 days for maturity. The number of species is an unsettled question, but there are no more than three. All forms have 20 chromosomes and cross readily. It varies in height from one to more than six feet. The growth varies from stiffly erect to prostrate.

The radicle forms a taproot with four longitudional rows of lateral roots arising from the pericycle. The cotyledons are fleshy and located on opposite sides of the stem. Each cotyledon has a bud in its axil that ordinarily remains dormant. The first true leaves are simple, opposite, and at right angles to the cotyledons. The other leaves are usually trifoliate and are located alternately on the stem. The cotyledons, primordia of the simple leaves, and the first trifoliate leaf primordia are located in the seed.

The flowers are commonly borne in axillary racemes. Flowers are typical for legumes. The fruit is a legume and has from one to five seed with two or more being common (Mische, 1961; Williams, 1960).

The soybean was the subject of early studies of plant flowering response to photoperiod. The seed, however, have no specific light requirement for germination. Soybeans are considered

to be quite sensitive to changes in environment. Light saturation for photosynthesis is thought to occur at an intensity of 2200 foot candles. Only 100 foot candles are required for flowering if the days are short.

Soybeans are a somewhat unusual crop in that there is ordinarily little response to nitrogen, particularly by well-nodulated plants (Howell, 1960). Soybeans are less tolerant to herbicides than most crops. Consequently, cultural practices including tillage and plant competition are commonly used to control weeds (Klingman, 1961).

Gross Effects of the Urea-Type Herbicides

The use of the substituted urea-type herbicides began with monuron (Bucha and Todd, 1951). Effects included top dieback, chlorosis, and growth retardation. Absorption of the herbicide was found to occur through the roots. Related compounds generally have similar effects. Cristoph and Fisk (1954) studied the anatomical effects of monuron on several plant species including soybeans. Soybeans were found to be very susceptible to rates greater than one-half pound per acre. Shoots died or became chlorotic; mature xylem of the stem was reduced; and compression and collapse of the cambium and disorganization of the phloem were striking. As there was a failure to produce normal amounts of secondary xylem and phloem, fundamental growth processes were thought to be affected. Wessels and Van Der Veen (1956) showed that there was little phloem translocation of monuron; although absorption through the roots and translocation to the shoots occurred readily. A number of compounds

of this type were studied and found to inhibit the Hill reaction of photosynthesis. Cooke (1956) reported the same result for monuron. Further proof of an effect on photosynthesis was offered by Gentner and Hilton (1960). They demonstrated that barley plants continued to live in the presence of lethal concentrations of fenuron (3-phenyl-1,1-dimethylurea), monuron (3-(p-chlorophenyl)-1,1-dimethylurea), diuron, and DMU (3-(3,4-dichlorophenyl)-1-methylurea) when fed sucrose through their leaf tips. It was thought that processes other than photosynthesis were involved, because sucrose was less effective at high concentrations of these herbicides. Hassal (1961) showed that the effect of monuron was most pronounced during metabolic activity. Contact between the herbicide and pea tissue in a refrigerator for long periods of time had little effect.

Though not a urea-type herbicide, DCMA [N-(3,4-dichlorophenyl) methacrylamide] reduced the enlargement of cotyledons, elongation of internodes, and expansion of true leaves of cotton (Bingham and Porter, 1961a). Microscopic examination of cotyledons and true leaves indicated that the rate and amount of cell enlargement was reduced by DCMA treatment. In addition, DCMA treatment appeared to immobilize the nitrogen of cotyledons.

Respiration of Plants

Respiration is the process in living organisms that releases the energy needed for maintenance and growth (Stiles, 1960). Two phases may be distinguished. The first part, or glycolysis, is the straight chain degradation of labile carbohydrate to the level of pyruvic acid (James, 1957). The oxidative phase is generally

considered to be the tricarboxylic acid cycle or the Krebs citric acid cycle. Electrons and protons produced by Krebs cycle activity are transported by the cytochrome system and united with oxygen to form water. Concurrently with these oxidations, released energy is stored as adenosine triphosphate (ATP) (Hackett, 1959). The respiratory process is considered to be essentially similar in plants, animals, and yeasts (James, 1957).

The localization of these processes in plant cells is of interest. James and Das (1957) showed that chloroplast preparations from broad-bean and spinach leaves exhibited no oxygen consumption; whereas mitochondrial preparations rapidly oxidized pyruvic acid and acids of the Krebs cycle. They suggested that carbon mobilization, glycolysis, and oxidation occurred in the chloroplasts, cytoplasm, and mitochondria respectively.

Respiration may be studied at various levels of organization in plants including intact plants, organs, sections, homogenates, or fractions such as mitochondria. The respiratory rate as measured by oxygen consumption may be very different in preparations that involve structural damage from that of the intact state (Green, 1959; Norris and Fohn, 1959).

The term "Terminal Oxidase" is applied to the enzymes that mediate the transfer of electrons to oxygen in the final stage of respiration. It has been recognized that three or more terminal oxidases exist. They are cytochrome oxidase, ascorbic acid oxidase, and polyphenol oxidase (Bonner, 1950).

Cytochrome Oxidase

The cytochromes were first reported by Keilin in 1925. In 1929 Keilin distinguished several components of the cytochromes including an indophenol oxidase. The activity of this oxidase could be inhibited by potassium cyanide, hydrogen sulfide, and carbon monoxide. Anything which affected the activity of this oxidase also affected oxygen uptake by cells. The indophenol oxidase is now known as cytochrome oxidase. The complete cytochrome spectrum was found to be present in the mitochondria of monocots and dicots by Hill and Bhagvat (1939). It is generally accepted that the respiratory chain terminates with the cytochrome system and that the cytochrome system is sufficiently active to handle the respiratory activities of plant tissues (Hackett, 1957). Most studies of this system have involved warm-blooded animals (Crane, 1961). A number of studies have described cytochrome oxidase activity in plants. Millerd, et al. (1951) were able to demonstrate cytochrome oxidase manometrically in mung bean. James and Boulter (1955) found cytochrome oxidase activity in barley roots that could be destroyed by boiling. Smillie (1956) was able to isolate mitochondria from green pea leaves that oxidized acids of the Krebs cycle and carried out oxidative phosphorylation. Eichenberger and Thimann (1957) thought that 80 per cent of the respiration of intact pea internodes was mediated by cytochrome oxidase. This was based on light reversible carbon monoxide inhibition of respiration. Crane (1957) reported active mitochondrial particles from cauliflower buds. Use of 0.001 molar versene was found to stabilize the particles for storage at -20

degrees C. Light and medium sized particles were distinguished. The medium sized particles appeared to be more active in succinate oxidation and were more sensitive to Antimycin A and cyanide. The light particles were insensitive to Antimycin A and appeared to have little cytochrome oxidase activity. Lundegardh (1958) studied the respiratory activities of various fractions from wheat root homogenates. Various types of particles were distinguished on the basis of size and activity. However, he concluded that "fractionation by means of centrifugation of homogenates does not effectively separate 'mitochondria' and 'microsomes'."

Sisler and Evans (1958a) studied the electron transport system of tobacco roots and concluded that the roots had sufficient cytochrome oxidase to account for respiratory oxygen uptake by the roots. Norris and Fohn (1959) were able to demonstrate cytochrome oxidase in onion root tips. The respiration of root homogenates was only four per cent of that of intact roots. It was also found that root sensitivity to carbon monoxide could be increased by depleting the root carbohydrate supply. Pierpoint (1959) extracted succinoxidase preparations from tobacco leaves with an extracting medium that included sucrose, tris buffer, phosphate, and ethylenediamine tetraacetate. Omission of any of these components reduced succinoxidase activity. Greater activity was found when cytochrome C, sucrose, and magnesium ions were added to the particle preparations. Wu and Scheffer (1960) followed a similar procedure and extracted particles from tomato leaves and stems. Particle preparations from young stems were more active than from leaves or older stems. Malate, succinate, fumarate, citrate, and α-ketoglutarate were

oxidized with coupled phosphorylation. Pyruvic acid was a poor substrate unless a catlytic amount of a Krebs cycle acid or "sparker" was added to the reaction mixture. Howell (1961) was able to demonstrate oxidation of Krebs cycle acids and oxidative phosphorylation by particles from etiolated soybean cotyledons by means of a similar procedure. Palmer (1961) demonstrated cytochrome oxidase in nutsedge tubers.

There have been reports of atypical cytochrome systems for some plants (Hartree, 1957). James and Elliot (1955) reported that mitochondria from the spadix of an Arum oxidized pyruvic acid and acids of the Krebs cycle. However, there was no inhibition by cyanide except to a limited extent for succinate. Bendall and Hill (1956) reported that the aroid spadix contained a normal cytochrome system in addition to the cyanide resistant fraction. The cyanide resistant fraction was attributed to the oxidation of a cytochrome designated as b_7, which is resistant to carbon monoxide and cyanide inhibition. Smith and Chance (1958) maintain that some of the b-type cytochromes can be auto-oxidized in the presence of cyanide.

Martin and Morton (1955) reported that microsomes from Silver beet showed a cyanide and Antimycin A resistant oxidation of reduced Coenzyme I and Coenzyme II. This was attributed to the presence of a cytochrome termed b_3.

Ascorbic Acid Oxidase

Szent-Gyorgyi (1930) reported that cabbage leaves contained an enzyme that catalyzed the oxidation of hexuronic acid. This acid later became known as L-ascorbic acid and the enzyme as ascorbic

acid oxidase. Ascorbic acid is recognized as being universally distributed in plants; although the oxidase may not always be present (Arnon, 1950). Ascorbic acid oxidase is specific for ascorbic acid; although the oxidation of ascorbic acid may be catalyzed by a number of other enzymes. Ascorbic acid oxidase is a copper specific protein according to Dawson (1950). Removal of copper with an ion exchange resin inactivated the enzyme (Joselow and Dawson, 1951). The enzyme is found only in higher plants and is insensitive to carbon monoxide (Bonner, 1957).

Inhibitors of ascorbic acid oxidase include cyanide, 8-hydroxyquinoline, and phenylthiourea according to Eichenberger and Thimann (1957). DDC (sodium diethyldithiocarbamate) and potassium ethylxanthate were listed as inhibitors by James (1953). James and Garton (1952) found that 0.2 mM DDC gave good inhibition of ascorbic acid oxidase and polyphenol oxidase with slight inhibition of cytochrome oxidase.

The localization of ascorbic acid oxidase is variable. Newcomb (1951) reported that it sedimented with the cell wall fraction from tobacco pith cells during differential centrifugation. Honda (1955) found it in the cell walls of barley roots. Bingham and Porter (1961) likewise found ascorbic acid oxidase in the cell wall fraction of cotton cotyledons. The 1,000 X G supernatant was found to contain the ascorbic acid oxidase of corn leaf tissue (Funderburk and Porter, 1961). Mertz (1961a) found ascorbic acid oxidase of corn roots to be in the cell wall fraction and in the soluble fraction; however, most (70%) was in the cell wall fraction. There

are reports that ascorbic acid oxidase may sometimes be localized in the mitochondria.

The role of ascorbic acid oxidase in plant respiration is far from being understood. A role as a terminal oxidase has been questioned because of its low affinity for oxygen (Bonner, 1957). The concentration of ascorbic acid and dehydroascorbic acid is highest in regions of high metabolic activity such as zones of elongation, floral buds, and leaf buds (Mapson, 1958). Ascorbic acid is not considered to be auto-oxidizable in the physiological range of pH so that the enzyme is required for its oxidation. A number of studies have indicated that there is some correlation between ascorbic acid oxidase activity of tobacco pith cells and cell wall growth. Mertz (1961a) found a striking parallel between the activity of ascorbic acid oxidase bound to corn root cell walls and root elongation. A role in cell wall growth separate from cellular respiration was suggested as the role for ascorbic acid oxidase. Newcomb (1960) showed that 5-15 X 10^{-4} M thiourea inhibited ascorbic acid oxidase activity and growth of tobacco pith sections while permitting a large respiratory increase with auxin.

James and Boulter (1955) reported that ascorbic acid oxidase replaced cytochrome oxidase in maturing barley roots. McWhorter and Porter (1960) reported ascorbic acid oxidase to be the predominant terminal oxidase in corn during the summer; however, the polyphenol oxidase became predominant later. Eichenberger and Thimann (1957) found an active ascorbic acid oxidase in homogenates of etiolated pea internodes that exceeded the respiratory rate of intact stems. Inhibitors indicated that the respiration of intact stems was mostly

mediated by cytochrome oxidase. They concluded that ascorbic acid oxidase was nonfunctional in intact cells as a terminal oxidase and was liberated by grinding. However, Bingham and Porter (1961b) found that cotton tissue was rather insensitive to carbon monoxide. Several substrates were tried with homogenates and ascorbic acid oxidase appeared to be the major terminal oxidase. This activity could be inhibited approximately 75 per cent with DDC and 35 per cent with thiourea.

Efforts have been made to demonstrate the participation of ascorbic acid and ascorbic acid oxidase in hydrogen transferring systems. A system involving triphosphopyridine nucleotide (TPN) and reduced glutathione (GSH) was reported by Mapson and Goddard (1951). A sequence of hydrogen transfer from substrates through TPN, glutathione, and ascorbic acid to oxygen was proposed. Alternate oxidation and reduction of glutathione would be required (Mapson and Moustafa, 1956). Mapson (1958) concluded that if ascorbic acid functions as a respiratory carrier, it is positioned between DPN or TPN and the terminal oxidase. Beevers (1954) maintained that the glutathione sequence must be dropped because TPNH cannot replace DPNH as a hydrogen donor. Crude cucumber extracts were able to rapidly oxidize DPNH when ascorbic acid was added. Monodehydroascorbic acid was proposed as the acceptor of electrons from DPNH. This system was linked to alcohol and alcohol dehydrogenases. Mertz (1961b) showed that the supernatant from corn root homogenates rapidly oxidized DPNH. Addition of ascorbic acid gave a 100 per cent stimulation, but dehydroascorbate had no effect. DDC and cyanide gave partial inhibition of DPNH oxidation.

Morrison, et al., (1961) reported that mitochondrial preparations carried out oxidative phosphorlation with ascorbic acid for a substrate. Addition of cytochrome C increased the oxidation of ascorbate.

Bingham and Porter (1961b) found that DCMA reduced the ascorbic acid oxidase activity of cotton to approximately the same extent that growth was affected. Funderburk and Porter (1961) found an atypical oxidation of ascorbic acid by preparations from corn leaf tissue that was unaffected by heat treatment and common inhibitors. DCMA treated plants showed an increased oxidation of ascorbic acid.

Polyphenol Oxidase

Boswell and Whiting (1938) are generally given credit for the discovery that polyphenol oxidases are involved in the respiration of higher plants. Polyphenol oxidases were found to be present in a majority of monocots and dicots. In the potato tuber, two-thirds of the oxygen uptake was mediated by a system that included an oxidase, a phenolic compound, and a dehydrogenase. Baker and Nelson (1943) found the same result for the potato.

The term polyphenol oxidase actually includes a group of enzymes that catalyze the oxidation of mono-phenolic and ortho-diphenolic compounds. Apparently the phenol oxidases are widely distributed in nature (Mallette, 1950). Li and Bonner (1947) studied the oxidase responsible for the blackening of tea leaves. It was found to be localized in the grana of chloroplasts and was insoluble in water. However, it was thought to have no role in the

respiration of tea leaves. A role in the darkening of plant tissues is commonly reported for the phenol oxidases (Mallette, 1950).

Mallette and Dawson (1949) studied the various properties of mushroom tyrosinase. Ultracentrifuge data indicated that it was a copper protein with four atoms of copper and a weight of about 100,000 per molecule. The polyphenol oxidases are inhibited by copper complexing agents such as DDC. The carbon monoxide inhibition of the polyphenol oxidases is not light reversible (James, 1953).

Palmer and Porter (1959a,b) showed that polyphenol oxidase was the dominant oxidase in dormant nutgrass tubers. Catechol was the most actively oxidized substrate. A later study by Palmer (1961b) has indicated that the oxidation of L-tyrosine by nutgrass tubers is essentially the same as for mammalian and cockroach tissues.

The functions of the polyphenol oxidases are somewhat uncertain. As mentioned previously, the respiration of some organs such as tubers may largely be mediated by polyphenol oxidases. It has been suggested that the polyphenol oxidases may be inactive in intact tissues. Injury to tissues would then activate these oxidases as evidenced by tissue browning. The production of materials that are toxic to foreign bodies may be another role for these enzymes (Arnon, 1950). It has been shown that ascorbic acid serves as a reducing agent for the oxidized product of L-tyrosine so that there is an equilibrium which maintains most in the intermediate form of 3,4-dihydroxyphenyl alanine. When the reducing system is disrupted, oxidation proceeds with the formation of melanin (Robinson and Nelson, 1944).

Catalase

Catalase is a metallo-protein enzyme with iron hematin as its prosthetic group. It is universally distributed in plants and animals. It serves to catalyze the breakdown of hydrogen peroxide so that toxic amounts do not accumulate in living tissues (Lardy, 1950).

Crystalline catalase was first prepared from beef liver by Sumner and Dounce (1937). It was the first iron-hematin containing enzyme to be isolated in a crystalline form and contained 0.1 per cent iron. One of the effects of iron deficiency in plants is to lower the catalase activity of plants (Weinstein and Robbins, 1955). Tourneau (1955) reported that wheat leaves infected with stem rust (Puccinia graminis tritici) at the urediospore stage showed increased catalase activity over the non-infected. The increased catalase activity was attributed to the catalase of spores.

Various chemicals have been found to affect the activity of animal and plant catalase. Landon (1934) found that several compounds including ammonium sulfocyanate and thiourea reduced the catalase activity of plants when supplied to the plants in water. Catalase activity of plants and animals has been reported to be reduced by amitrol (Heim, et al., 1956; Pyfrom, et al., 1957). Extracts from rat cancers have shown a similar effect on catalase (Heim, et al., 1956).

Palmer and Porter (1959c) reported that germinated nutgrass tubers exhibited a catalase activity 18.5 times higher than that of

tubers treated with 8 mg of amitrol. Bingham and Porter (1961c) reported that the catalase activity of cotton cotyledons that were treated with DCMA remained constant while that of control tissue increased. Catalase activity of corn tissue was reduced by DCMA treatment (Funderburk and Porter, 1961).

Peroxidase

Peroxidase is an enzyme of universal occurrance in plants (Bonner, 1950). It probably catalyzes the oxidation of phenols and aromatic amines by hydrogen peroxide (Lardy, 1950). This idea has been questioned in one instance. Yamazaki, et al. (1957) suggested a scheme of hydrogen transfer where oxygen was reduced to hydrogen peroxide and then further reduced to water. This process would be mediated by peroxidase. Whatever the correct scheme may be, peroxidase contains iron hematin as the prosthetic group and is inhibited by cyanide and other inhibitors of iron-containing enzymes.

Heim, et al. (1956) found that amitrol temporarily reduced the activity of rat liver peroxidase. Palmer and Porter (1959c) found that amitrol maintained the peroxidase level of nutgrass tubers at the dormant level. The level of germinated tubers was 2.5 times that of dormant or treated tubers. Bingham and Porter (1961c) found that peroxidase activity of DCMA treated cotton cotyledons was unchanged while that of control tissue increased. A reduction in the level of peroxidase activity of DCMA treated corn leaf tissue preparations was found by Funderburk and Porter (1961).

Glycolic Acid Oxidase

Claggett, et al. (1949) reported the discovery of an enzyme that catalyzed the oxidation of α-hydroxy acids. It was found to be common in green leaves but was absent from embryos and etiolated seedlings. It has since become known as glycolic acid oxidase, although L-lactic acid will also serve as a substrate. A pH optimum of 7.6 was found for lactate oxidation and an optimum between 7.8 and 8.6 for glycolate. Tolbert, et al. (1949) partially purified the enzyme and found that lactate was oxidized to pyruvate and hydrogen peroxide, whereas glycolate was oxidized to glyoxylate and hydrogen peroxide. Kun (1952) found that rat liver preparations oxidized glycolic acid to glyoxylic acid, and he thought that the enzyme was a flavoprotein. Zelich and Ochoa (1953) isolated the enzyme from spinach in a highly purified form and found the prosthetic group to be riboflavin monophosphate. In the absence of catalase, glycolate was oxidized from glyoxylate to formate, water, and carbon dioxide and lactate from pyruvate to acetate, water, and carbon dioxide. The oxidation of lactate occurred at about one-half the rate of glycolate oxidation.

On the basis of inhibitor studies, Noll and Burris (1954) concluded that the enzyme depends on sulfhydryl groups for activity. Frigerio and Harberry (1958) made an exhaustive study of purified glycolic acid oxidase from spinach leaves. After various tests, they concluded that the prosthetic group was not a metallo-flavoprotein and only participated in two-electron oxidation-reduction

systems. Noll and Burris (1954) reported that etiolated plants were not devoid of glycolic acid oxidase, but that the enzyme activity was greatly enhanced by illumination.

Inhibitors of glycolic acid oxidase include oxalic acid, mesooxalic acid, hydrazine, semicarbazide, ethylene diamine, and ascorbic acid (Tolbert, et al., 1949). Zelich (1957) reported that α-hydroxysulfonates were effective competitive inhibitors of glycolic acid oxidase. Acetaldehyde and formaldehyde bisulfites were nearly equally effective at concentrations of one per cent those of lactate and glycolate.

Zelich (1953) reported the isolation of a pyridine nucleotide linked glyoxylic acid reductase. In the presence of glycolic acid, glyoxylic acid, and pyridine nucleotides, glyoxylic acid reductase and glyoxylic acid oxidase acted as a hydrogen transferring system. It was suggested that this system might function in the respiration of green leaves.

A relationship between photosynthetic activity and glycolic acid oxidase seemed evident with the discovery of the enzyme. Zelich (1958) showed that there was a ten-fold increase in the leaf concentration of glycolic acid within one hour in the presence of glyoxylate bisulfite. Other evidence indicated that one-half of the carbon fixed in photosynthesis may be metabolized by this enzyme (Zelich, 1959). As no increase was found in darkness, it was thought that glycolic acid is a product of photosynthesis. Griffith and Byerrum (1959) found that Nicotiana rustica leaves synthesized glycolic acid, glycine, serine, and alanine from

D-ribose-1-C^{14}. The labeled carbon was principally in C_1 of glycolic acid, which probably gave rise to glycine and serine. Moses and Calvin (1959) found that glycolic acid was formed within five seconds in experiments with tritiated water. As no labeled glyoxylate was observed, it was suggested that glycolate serves as a hydrogen transferring agent.

Glycolic acid oxidase is thought to be primarily localized in the chloroplasts (Moses and Calvin, 1959), although some may be found in particulate preparations (Zelich and Barber, 1960). Zelich and Barber (1960) were unable to link any significant amount of phosphorylation with glycolic acid oxidation.

All evidence indicates that glycolic acid oxidase synthesis or activity increases with leaf age (Noll and Burris, 1954; Zelich, 1958). There have also been reports that the Krebs citric acid cycle decreases in activity with age (Daly and Brown, 1954; Zelich and Barber, 1960). This has caused some investigators to think that glycolate activity suppresses the Krebs cycle in maturing leaves (Zelich and Barber, 1960). In fact, D'Abramo, et al. (1958) have maintained that glyoxylate combines with oxaloacetate to form oxalomalate, which inhibits the oxidation of citric acid.

Another pathway that involves the oxidation of glycolic acid is the glyoxylate cycle or shunt. This is a variation of the Krebs cycle that seems to be functional in the conversion of fats in storage organs into carbohydrates (Kornberg and Beevers, 1957). In brief the process is as follows:

Fat → fatty acyl Co A → acetyl Co A → [glyoxylate cycle] → malate → phosphoenolpyruvate → [glycolysis] → carbohydrate

Briefly, the glyoxylate shunt is as follows:

2 isocitrate → α-ketoglutarate + glycolate + succinate + CO_2

Acetyl Co A + Glyoxylate → malate + Co A.

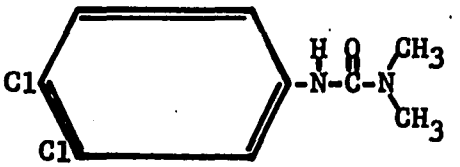
Bingham and Porter (1961c) found glycolic acid oxidase to be present in cotton cotyledons, but it was not affected by DCMA treatment. Funderburk and Porter (1961) found that there was a 50 per cent reduction in glycolic acid oxidase activity from corn tissue six days after DCMA treatment.

METHODS AND MATERIALS

I. The Herbicide

The herbicide used in these experiments was General Chemical-6691 or DIURON-DBSA. It contains 33.54 per cent of DIURON-DBSA by weight or three pounds per gallon. There are 1.25 pounds of diuron per gallon.

Diuron is the common name for 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, which was introduced by the E. I. du Pont de Nemours & Co. as an experimental herbicide in 1955. It is rather stable to oxidation and moisture. The maximum solubility is only approximately 42 pp. It is a general herbicide and shows selective toxicity in certain crops at rates of 0.6 to 4.8 pounds per acre. The structural formula is as follows:



DBSA stands for dodecyl benzene sulfonate, a detergent with the formula of $C_{12}H_{25}C_6H_4SO_3Na$.

II. Growth of Plant Material

Lee soybeans were used as the source of experimental material in all experiments except the rice bioassay. Lee is one of the better commercial varieties of soybeans grown in the South (Hartwig, 1962).

In most experiments, soybeans were planted in the greenhouse in flats of a sandy loam soil. On the eighth day after planting,

one-half of the flats were sprayed with DIURON-DBSA at the rate of three-eights of a pound per acre (0.16 pound per acre of diuron). The flats were moved to a growth chamber with a temperature of 85 degrees Fahrenheit and a photoperiod of 20 hours. Light intensity was approximately 500 foot-candles. Enzyme activities were ordinarily determined on one, four, and seven days after herbicidal treatment.

In some experiments, soybean seed were germinated in a moist chamber and transferred to 250 ml Erlenmeyer flasks containing Hoagland and Arnon's nutrient solution number two (Hoagland and Arnon, 1950). After the plants had grown for several days, the nutrient solutions were replaced with solutions that contained logarithmic concentrations of DIURON-DBSA.

In some experiments, the soybeans were transplanted so that the lower portion of their roots were growing in nutrient solution and the upper portion in soil. After the plants became established, some nutrient solutions were changed for solutions with lethal concentrations of DIURON-DBSA to determine if the effect was through root injury or injury to the shoots of the plants.

In experiments with cytochrome oxidase, seed were germinated in a darkroom in refrigerator drip trays. The trays were lined with paper toweling and moistened with distilled water. Bean seed were planted in rows formed by grooves in the bottoms of the trays. To one tray was added 40 ml of 100 ppm diuron as DIURON-DBSA (5 ml X 8 grooves). The beans were covered with paper toweling and watered each day with distilled water. Particulate preparations were made on the ninth day after the beginning of germination.

III. Growth Tests

Some effects of DIURON-DBSA on treated and control plants were measured by determining fresh weights of simple leaves of treated and control plants. At the same time, lengths of epicotyls were measured.

The effect of DIURON-DBSA in the rice bioassay was determined. Two sheets of filter paper were placed in a Petri dish. Five ml of herbicide with concentrations varying from 0 to 500 ppm were added according to the treatment. Twenty rice seed were planted in each dish with three replicates or dishes per treatment. Dishes were covered, labeled, and placed in an incubator at 85 degrees Fahrenheit for four days. At the end of the bioassay, germinated seed were counted and the lengths of the radicles were determined.

IV. Preparation of Tissue for Respiratory Experiments

In respiratory experiments, plants were grown in the greenhouse until the sixth day. At that time, plants were moved to the darkroom or to the growth chamber. On the eighth day, one-half of the plants were sprayed with herbicide. Plants were returned to the darkroom or growth chamber. This gave a total of eight treatment combinations. They can be represented by letting L and D stand for light and dark pre-treatment and 1 and d stand for light and dark after-treatment:

L-UT-1	D-UT-d
L-UT-d	D-UT-1
L-T-1	D-T-d
L-T-d	D-T-1

In respiratory determinations, disks were cut from simple leaves with a cork borer. To each Warburg flask were added 100 mg of disks. Oxygen consumption and carbon dioxide evolution were determined for each treatment. At the end of an experiment, the disks were digested for nitrogen determination.

V. Preparation of Homogenates and Supernatants

Most experiments involved simple leaves, although cotyledons were used in some studies. Midribs were removed from the leaves so that the homogenates could be transferred more easily with pipettes. Two grams of leaf slices were placed in a Servall Omni-mixer with 10 ml of 0.067 M phosphate buffer. The sample was homogenized for 10 minutes at 85 volts. During homogenation, the chamber of the homogenizer was placed in an ice bath at less than five degrees C. The contents were rinsed from the chamber and buffer was added to give a final volume of 30 ml.

VI. Preparation of Particles

Particles were prepared according to the procedure given by Wu and Scheffer (1960). Thirty grams of stems or cotyledons were minced and chilled for 10 minutes at 2 degrees C. This was followed by grinding in a pre-chilled motar with 15 grams of sand and 60 ml of extracting solution for two minutes. The macerated tissue was strained through six layers of cheesecloth and centrifuged for 10 minutes at 2 degrees C. The supernatant was centrifuged in the Spinco Model L ultracentrifuge at 10,000 X G for one-half hour. The supernatant was decanted and the "particles" were resuspended

in 30 ml of washing solution. The particles were again centrifuged at 10,000 X G for one-half hour. The particles were suspended in 10 or 20 ml of 0.25 M sucrose solution for enzyme determinations. The composition of the solutions are given below.

	Extracting Solution	Washing Solution
Sucrose	0.4 M	0.2 M
Tris-Buffer	0.2 M	0.1 M
Sodium EDTA	0.005 M	0.0025 M
Dibasic Phosphate	0.01 M	0.0005
Sodium Citrate	0.02 M	0.0005 M
Adjust pH to:	7.9	7.3

VII. Determination of Oxygen Uptake and Carbon Dioxide Evolution

Gas exchange was determined manometrically according to the methods of Umbreit et al. (1957). The temperature of the water bath was 25 degrees C unless stated otherwise. One to two ml of enzyme preparation were usually added to the main chamber of the Warburg flasks, and the substrate was placed in the sidearm. A 2 X 2 cm folded square of Whatman No. 1 filter paper and 0.15 ml of 20 per cent sodium hydroxide were placed in the center well to absorb any carbon dioxide. Duplicate flasks were used for each treatment.

When carbon dioxide evolution was determined, pairs of flasks were used. One had a piece of filter paper and base in the center well; whereas the other had none. Any carbon dioxide evolved during progress of the experiment could be calculated by the indirect method (Umbreit, et al., 1957).

Flasks were allowed to equilibrate for 10 minutes before closing the stopcocks. For some enzymes, endogenous respiration

was determined for 15 minutes before tipping the substrate into the main chamber of the flasks. Manometers were ordinarily read at fifteen minute intervals for one hour and hourly at two and three hours.

Results were expressed as microliters of oxygen or carbon dioxide consumed or evolved per milligram of amino nitrogen per hour [QO_2 (NH_2) or Q CO_2 (NH_2)]. Some results were expressed as microliters of oxygen consumed or evolved per gram of fresh weight or per leaf per hour [QO_2 (fr wt) or QO_2 (leaf)]. Other results were expressed as microliters of oxygen consumed per milligram of amino nitrogen [$\mu l\ O_2$/ mg NH_2] or per leaf [$\mu l\ O_2$/ leaf].

VIII. Estimation of Enzyme Activity

A. Cytochrome Oxidase

Cytochrome oxidase activity was determined by the oxidation of succinate. Four-tenths ml of particles plus 300 μM sucrose, 140 μM phosphate, 15 μM $MgSO_4$, 0.2 μM cytochrome C, and 50 μM of succinate were added to the main chamber of each flask. The reagents were prepared so that 0.2 ml contained the required amount of each. Buffer or inhibitor in buffer were added to give a volume of 2.0 ml excluding the volume of paper and base. The pH of the various reagents was adjusted to pH 7.3. A temperature of 30 degrees C was used.

Pyruvic and some other acids of the Krebs cycle were used as substrates. The procedure followed for these was that of Wu and Scheffer (1960). Flasks were allowed to equilibrate for 10 minutes for all substrates before closing the stopcocks. Manometers were read at 15 minute intervals for one hour.

B. Ascorbic Acid Oxidase

Ascorbic acid oxidase activity of soybean leaf homogenates was rather high so that only one ml of the whole homogenate was added to each flask. One-half ml of 0.25 M ascorbic acid was added to the sidearm of each flask. All solutions were prepared in 0.067 M phosphate buffer and the pH values adjusted to 6.0.

After a ten minute equilibration period, the stopcocks were closed. Endogenous respiration was determined for 15 minutes and the substrate was tipped into the main chamber. Manometer readings were made at 15 minute intervals for one hour.

C. Polyphenol Oxidase

Two ml of enzyme preparation were added to the main chamber of the Warburg flasks and one-half ml of 0.12 M catechol was added to the sidearm. Also, 1.35 ml of buffer or inhibitor were added to the main chamber. After an equilibration period of ten minutes, the stopcocks were closed and manometers were read at 15 minute intervals for one hour and at the end of two and three hours. All solutions were adjusted to pH 6.0 and the temperature was 25 degrees C.

D. Glycolic Acid Oxidase

A homogenate supernatant of 1,000 X G was used for these studies. Two ml of enzyme preparation were added to the main chamber of each Warburg flask, and one-half ml of 0.14 M glycolic acid was added to the sidearm. A volume of 0.675 ml of buffer or inhibitor in buffer was added to the main chamber. All solutions were pH 7.8 and the water bath temperature was 25 degrees C. Manometer stopcocks were closed after a ten minute equilibration

period and endogenous respiration was determined for 15 minutes. The substrate was tipped into the main chamber and oxygen uptake was recorded at 15 minute intervals for the first hour and at the end of two and three hours.

E. Catalase

A modification of the procedure of Appleman (1951) was used for the determination of catalase activity. Two ml of hydrogen peroxide (final concentration of 10 mM) were added to the main chamber of the Warburg flask. One ml of enzyme preparation (ten-fold dilution of the 1,000 X G supernatant) was added to the side-arm. All solutions were made in 0.067 M phosphate buffer of pH 7.0 and the water bath temperature was 20 degrees C. After a ten minute equilibration period, the enzyme was tipped into the main chamber and oxygen production was determined at one minute intervals for 10 minutes.

F. Peroxidase

The general procedure for peroxidase determination followed that of Ettori (1949). The enzyme source was a 1,000 X G supernatant. To the main chamber of each Warburg glask were added 0.2 ml of 5 per cent pyrogallol, 1.5 ml of water, and 0.2 ml of one per cent hydrogen peroxide. To the sidearm were added 0.2 ml of enzyme and 0.5 ml of 0.25 M phosphate buffer. After an equilibration period of ten minutes, the contents of the sidearm were tipped into the main chamber and manometers were read at one minute intervals for 10 minutes. Two sets of flasks were used for each treatment. One set contained paper and alkali, whereas the other had only the

other reagents. Carbon dioxide production was calculated by the indirect method (Umbreit, et al., 1957).

IX. Inhibitor Studies

Several inhibitors were used for studying the properties of the various types of enzymatic activity. The contents of Warburk flasks were constant in volume and included the enzyme source, substrate, base, filter paper, buffer, and other materials. When inhibitors were used, part of the buffer volume was replaced by a concentrated inhibitor solution. This usually involved a five-fold dilution to give the desired final inhibitor concentration.

X. Nitrogen Determination

Amino nitrogen determinations were made by the Kjeldahl procedure of Hiller, et al. (1948). One or two ml of enzyme preparation were added to a Kjeldahl flask with a granule of copper selenate catalyst. To this was added eight ml of digestion mixture (500 ml water + 75 g Na_2SO_4 + 500 ml of concentrated reagent sulfuric acid). The sample was digested with moderate heat until clear. Samples were allowed to cool and 25 ml of water were added. Flasks were closed and stored in a refrigerator until distillation of the samples.

Ammonia was distilled from samples by means of a micro-Kjeldahl distillation apparatus. The sample was added to the distillation chamber and followed by 15 ml of 40 per cent (weight/weight) sodium hydroxide. Steam bubbles were started in the distillation chamber and a 50 ml Erlenmeyer receptor flask was

raised so that the tip of the delivery tube was covered by the 10 ml of 2 per cent (weight/volume) boric acid solution. Two drops of methyl red were added to each flask. Distillation was continued until the indicator had changed from red to yellow. The receptor flask was lowered when approximately one-half full and the delivery tube allowed to drip until the flask was nearly two-thirds filled. The delivery tube and distillation chamber were washed with cold distilled water between samples by means of back suction into a water trap.

The samples were back titrated with approximately 0.01 N sulfuric acid (0.245 ml of concentrated reagent grade sulfuric acid per liter) to the original red color. The exact normality of each batch of sulfuric acid was determined by titration against a standard sodium hydroxide solution. The nitrogen factor (mg N/ml of acid) was calculated by multiplying the atomic weight of nitrogen by the normality of the acid. The number of mg of N per sample is equal to the product of ml of acid used in titration and the nitrogen factor for the acid. Duplicate nitrogen determinations were made for each treatment.

XI. Preparation of Permanent Slides and Photomicrographs

Observation had indicated that the herbicide reduced the growth and development of the bean plants. It was decided to study this further by means of permanent slides from treated and control plants. Sections from primary leaf midribs, epicotyls, and roots were cut from plants on the fourth day after herbicide treatment and placed in FAA killing and fixing solution (2.3%

acetic acid + 6.7% of 40% formaldehyde + 91.0% of 50% ethyl alcohol). Vials containing sections were placed in a vacuum desiccator to get rapid killing and fixing.

Killed samples were washed in 50 per cent ethanol three times and transferred through a series of alcohol solutions to pure tertiary butyl alcohol. This was replaced by a solution made of equal amounts of tertiary butyl alcohol and paraffin oil. The samples were then poured over partially solidified paraffin, placed in a paraffin oven, and the samples were allowed to settle to the bottoms of the containers. This was followed by three changes of Gulf paraffin and tissuemat. Samples in tissuemat were left in the oven for one-half hour before molding into blocks.

Small blocks with individual samples were mounted on sample holders, and serial sections were cut on an American Optical rotary microtone. Six sections were mounted on each slide with Haupt's fixative.

Tissuemat was removed from the sections by "running the slides down" through a series of solutions from pure xylene to safranin in water. Slides were transferred through a series of solutions to pure ethanol, stained with fast green, cleared with clove oil, and "run up" to pure xylene. Cover slips were mounted on the sections with balsam and the slides were allowed to dry. Sections were also stained with orange G and gentian violet but did not give satisfactory photomicrographs.

Examination of slides under a microscope showed that the simple leaves were most affected by the herbicide treatment. Leaf

midrib cross-sections were photographed on 3¼ X 4¼ Kodak M plates by means of a Bausch and Lomb photomicrographic camera. A green filter was used with the Ortho illuminator to increase the contrast between safranin and fast green stained portions of sections.

EXPERIMENTAL RESULTS AND DISCUSSION

I. Effects of DIURON-DBSA on Early Growth and Development

It is of interest to compare the effect of DIURON-DBSA with 2,4-dichlorophenoxyacetic acid (2,4-D) in a bioassay. The rice bioassay was used because the rice root radicle is rather sensitive to auxin-type compounds. One ppm 2,4-D gave almost complete inhibition of the rice root radicle (Figure 1). The DIURON-DBSA concentration that gave a comparable inhibition was 100 ppm (Figure 2). Rice seeds germinated at the highest 2,4-D concentration, but no germination was found for DIURON-DBSA at 500 ppm. It is apparent that 2,4-D has more pronounced auxin properties.

After it had been decided to use DIURON-DBSA as an experimental herbicide, several rates of application were tried with soybeans. A rate of three-eighths pound per acre was eventually selected because treated leaves lived for a week. This allowed time to follow the various effects of the herbicide on the treated plants. Herbicide injury symptoms usually appeared by the fourth day. The herbicide was applied on the eighth day after the start of germination, because the simple leaves had expanded sufficiently to intercept the herbicide.

It was thought at first that the herbicide was translocated from the site of application to the other portions of the shoots. This was disproved by bagging and dipping experiments. Several plants in a flat of beans were marked with string. One simple

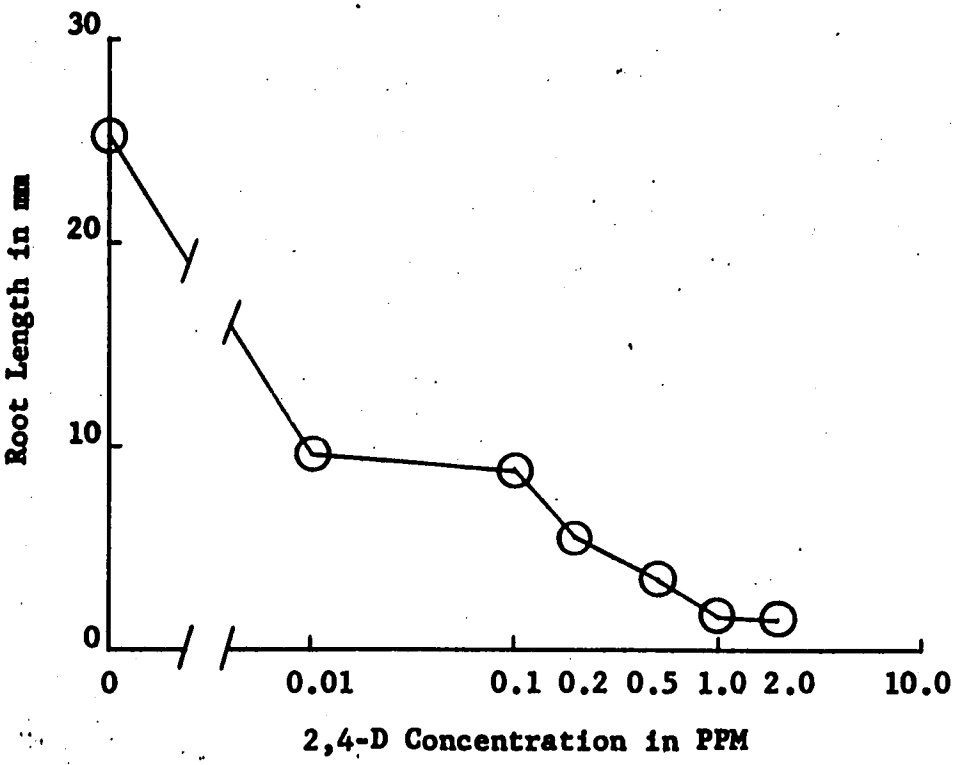


Figure 1. The effect of varied concentrations of 2,4-D on elongation of the rice root radicle.

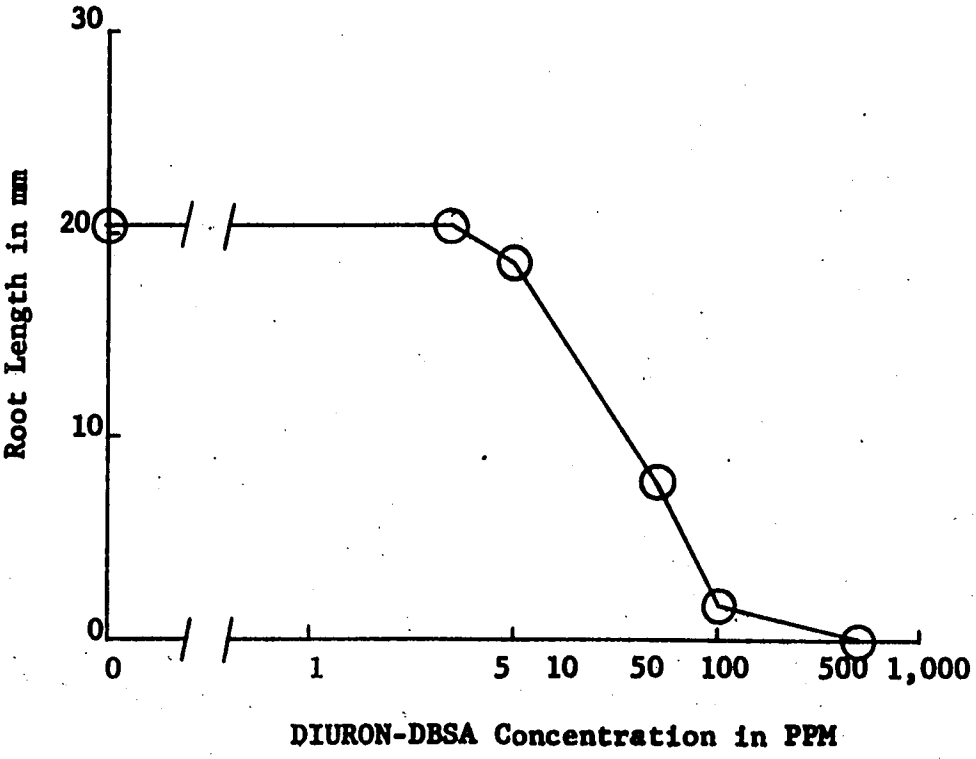


Figure 2. The effect of varied concentrations of DIURON-DBSA on elongation of the rice root radicle.

leaf from each plant was covered with a cellophane bag and the flat was sprayed in the usual manner. It quickly became obvious that the protected leaves were unaffected unless the stems of the plants were killed by the herbicide. Dipping of individual leaves in a solution of 760 ppm DIURON-DBSA showed that only the treated leaves were affected. This is in agreement with the belief that there is little, if any, phloem translocation of the substituted urea herbicides (Klingman, 1961; Wessels and Van Der Veen, 1956).

It was decided to determine if the herbicide was absorbed by the roots and translocated to the tops to kill the plants. Soybean seed were germinated in darkness and the seedlings were transferred to 250 ml Erlenmeyer flasks containing aerated nutrient solution. After eight days, the solutions were changed for nutrient solutions containing 0, 1, 10, 100, and 1,000 ppm DIURON-DBSA. All plants were dead except for the check and one plant at 1 ppm within three days. The experiment was repeated with concentrations of 0, 0.1, 1.0, and 10 ppm. All plants were dead or dying at concentrations above 0.1 ppm within four days after the initiation of herbicide treatment.

It was suggested that the effect of the herbicide could be caused by root injury rather than direct injury to shoots of the plants. A split-root experiment was devised to test this idea. Soybeans were grown for four days in nutrient solution and partially transplanted to small pots of soil so that the lower portions of their roots could grow in nutrient solution. Both

soil and nutrient solution water losses were replaced with plain nutrient solution. The root systems of four plants continued to live with the split arrangement. After four days, nutrient solutions were changed so that two flasks contained 10 ppm DIURON-DBSA in nutrient solution, whereas the other two had plain nutrient solution. Plants with their lower root systems exposed to the herbicide solution died within five days, while the other two remained alive. The apparent ready absorption of the herbicide through the soybean roots followed by top kill agrees with the literature on this point (Bucha and Todd, 1951; Klingman, 1961; and Wessels and Van Der Veen, 1956).

There may be a question as to the susceptibility of shoots in comparison with roots. Hassall (1961) reported that pea shoots grown in phosphate buffer were no more susceptible to monuron than roots. He maintained that the reported susceptibility of shoots could be explained on the basis that monuron was accumulated in the shoots by transpiration.

Attempts to produce top kill by application of the herbicide to soil were less successful. Despite removing cotyledons and simple leaves, development of chlorosis was slow. Rates of 10, 1, 0.1 and 0 ppm DIURON-DBSA in water were applied daily to soil from the twenty-third day. All plants receiving 10 ppm were dead by the forty-seventh day. Plants receiving 1 and 0.1 ppm showed some chlorosis. This most likely indicates that the herbicide was fixed by soil colloids.

Gentner and Hilton (1960) published evidence which indicated that the feeding of sucrose to barley plants that had been treated

with the substituted ureas increased the plants' resistance to the herbicides. It was decided to determine if light and dark treatments would affect the response of the beans to the herbicide. The development of chlorosis by trifoliate leaves that had been dipped into a 760 ppm DIURON-DBSA solution is graphically depicted in Figure 3. It can be seen that the leaves became chlorotic as quickly when the herbicide was applied after two days of darkness as when applied at the beginning of the dark period. Complete chlorosis never developed for treated leaves of plants that were left in the growth room during the entire experimental period. Treated leaves became chlorotic when the herbicide was applied after two days of darkness even though treatment was followed by light treatment. The development of chlorosis was apparently related to the depletion of the leaf photosynthate level. Data are not presented for longer periods than four days, because the check plants were becoming etiolated in the darkroom.

An attempt was made to feed sucrose through cut tips of middle leaflets of herbicide treated trifoliate leaves. The results were inconclusive for this experiment. The arrangement of the conduction tissue in soybeans may be such that insufficient sucrose was absorbed to prevent chlorosis.

It was observed that the heights of treated plants were less than those of the untreated plants (Figure 4). Study of stem growth by segments during the seven-day testing period revealed that there was some reduction in the elongation of the internode between the cotyledons and the primary leaves (Figures 5, 6). However, the

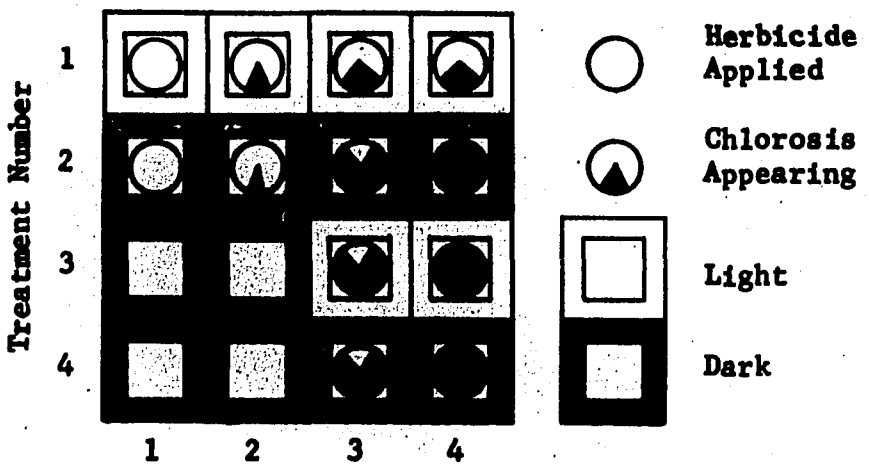


Figure 3. The effect of light on the appearance of chlorosis in DIURON-DBSA treated trifoliate leaves.

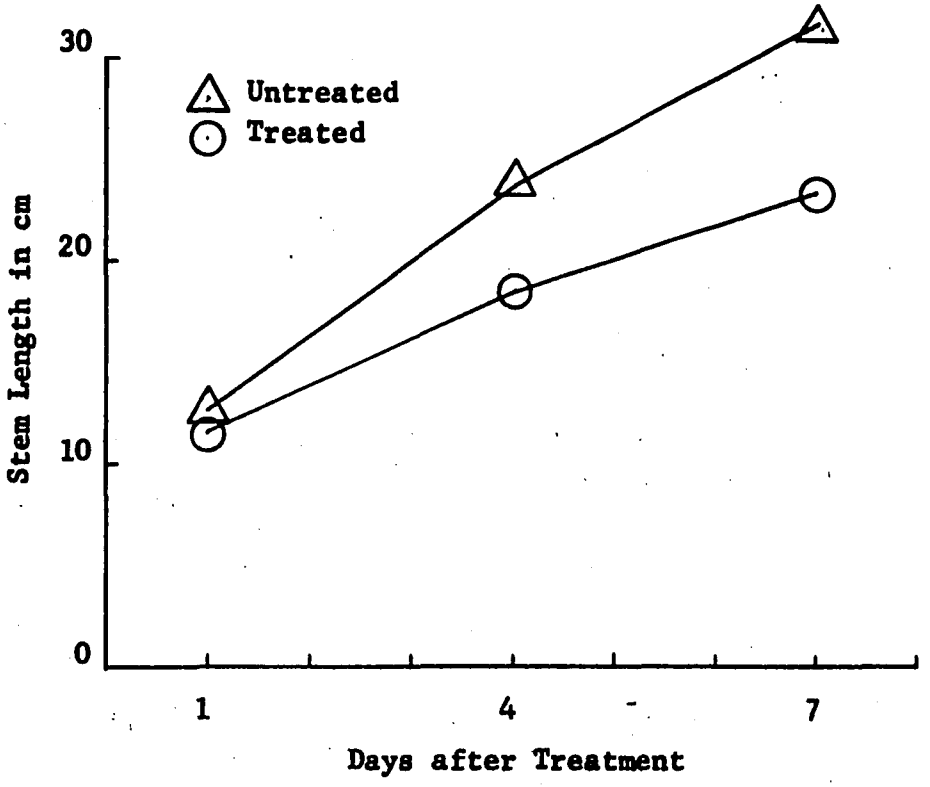


Figure 4. The effect of DIURON-DBSA treatment on stem elongation from the soil to the first trifoliate leaf.

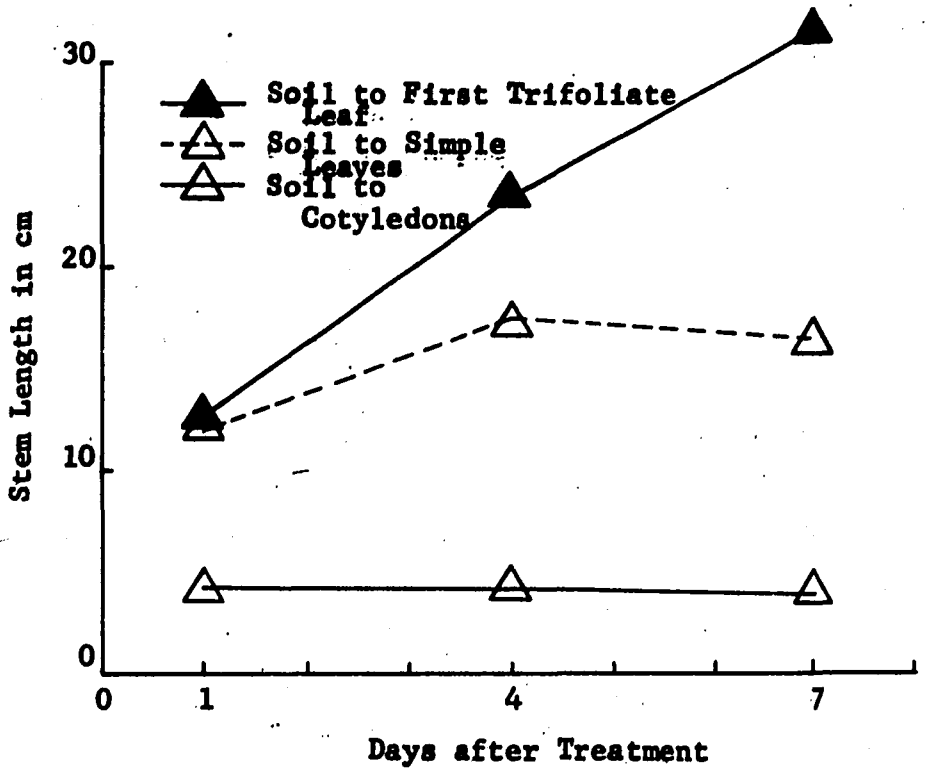


Figure 5. The growth of untreated stems by sections during the seven-day testing period.

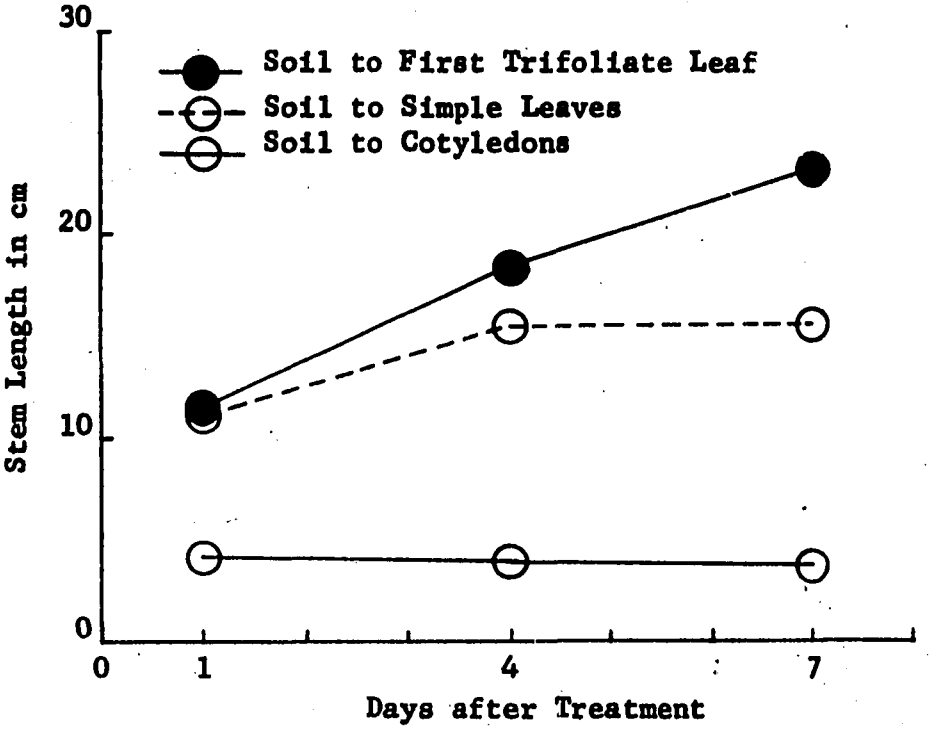


Figure 6. The growth of DIURON-DBSA treated stems by sections during the seven-day testing period.

greatest reduction was for the internode between the primary leaves and the first trifoliate leaf. It was notable that the stem length from the ground to the cotyledons was unaffected by the herbicide treatment. The plants involved in these measurements were measured only once because they were removed from the flats for leaf weight determinations. Despite the use of different plants for each series of measurements, these results were remarkably consistent. It was also observed that the cotyledons of treated plants usually remained green for a longer period of time than for untreated plants (Figure 7). This possibly indicates that the mobilization of food reserves from cotyledons was reduced by the herbicide treatment.

The effect of the herbicide on the fresh weights of simple leaves was also determined. It may be noted in Figure 8 that there was less increase in leaf fresh weight with herbicide treatment. The same trend was evident for weights of entire leaves or for leaves without midribs. Bingham and Porter (1961a) reported a similar effect of DCMA on cotton cotyledons.

The data on leaf amino nitrogen content may be rather significant. Examination of Figure 9 indicates that there was little difference in the total amino nitrogen per leaf for treated or control leaves. Also, the total amino nitrogen per leaf did not change during the experimental period. Apparently, the leaf amino nitrogen content became stationary before herbicide treatment.

Examination of Figure 10 indicates that the leaf nitrogen concentration (mg N/ gm fresh weight) decreased during the experimental period, particularly for the control treatment. This is

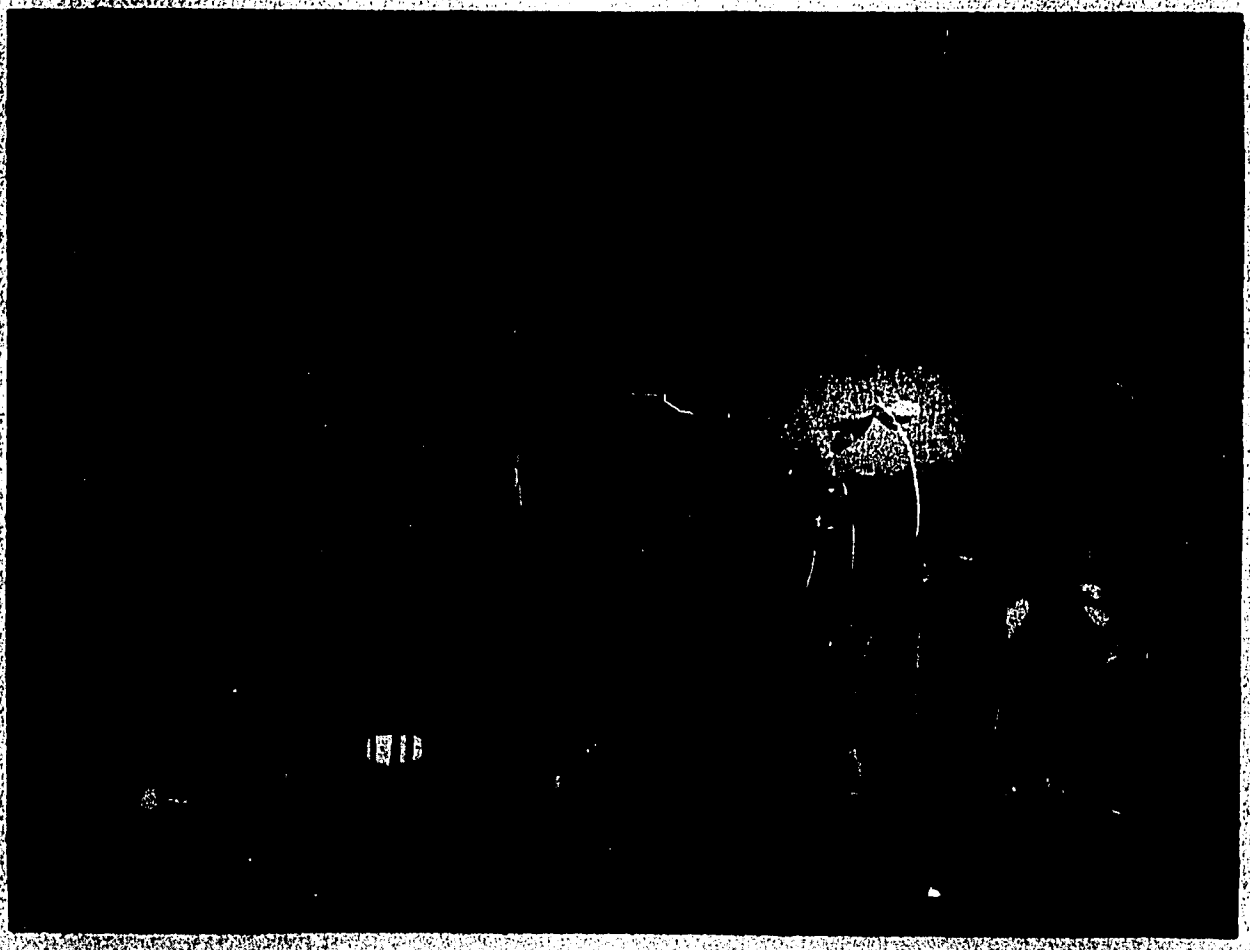


Figure 7. A tray of untreated beans (left) compared with a tray of treated beans (right) on the seventh day after treatment with DIURON-DBSA at the rate of three-eights pound per acre.

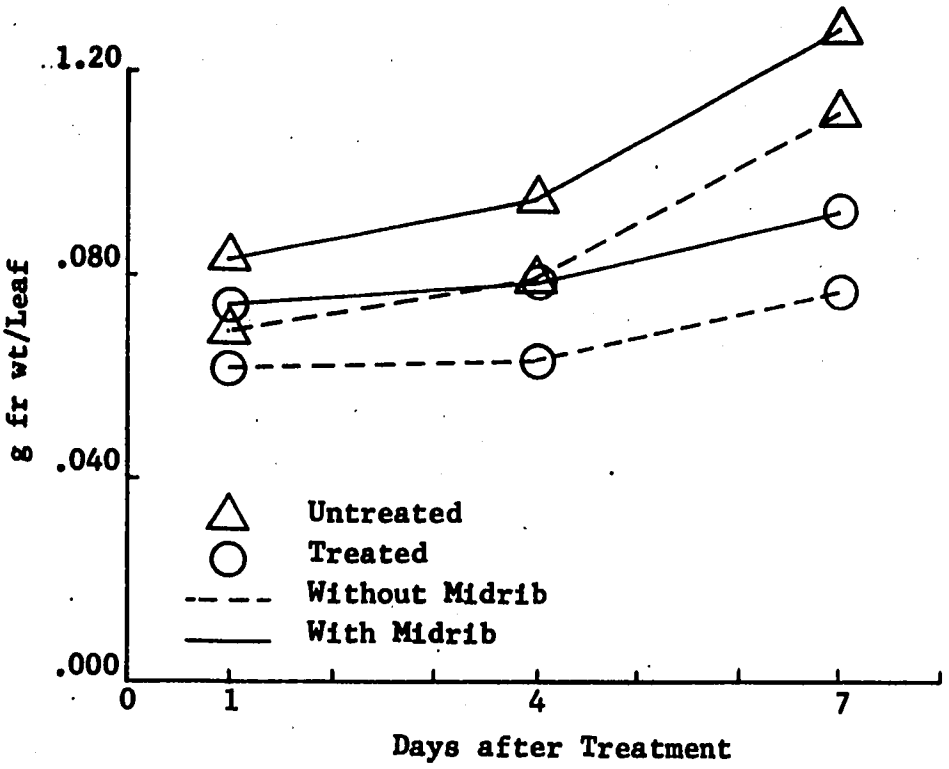


Figure 8. The effect of DIURON-DBSA on the fresh weight of soybean primary leaves.

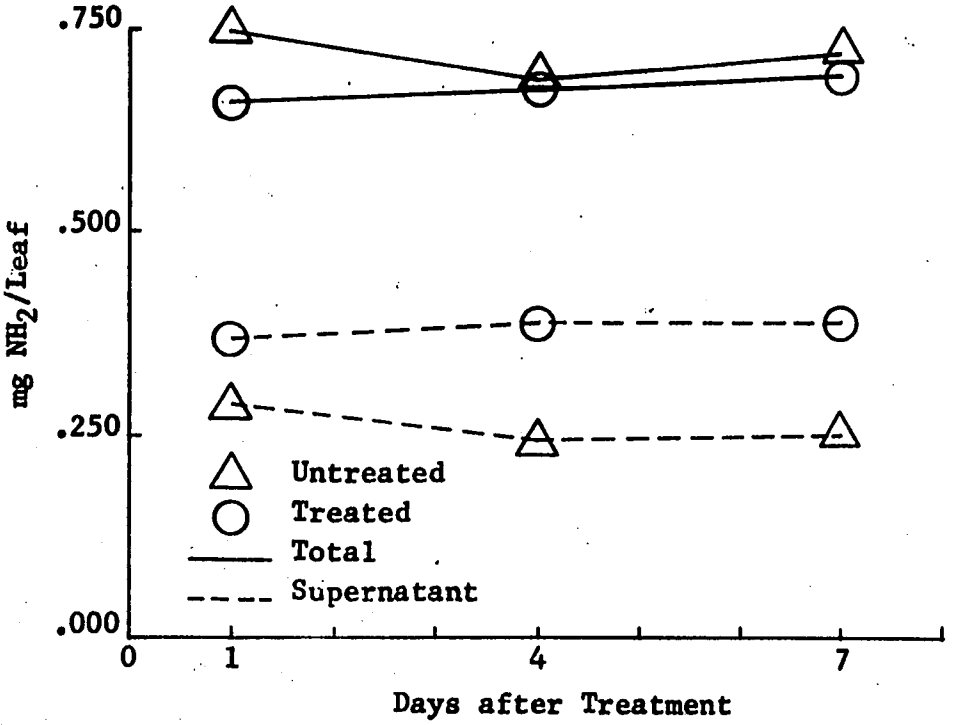


Figure 9. The effect of DIURON-DBSA on the amino nitrogen content of primary leaves.

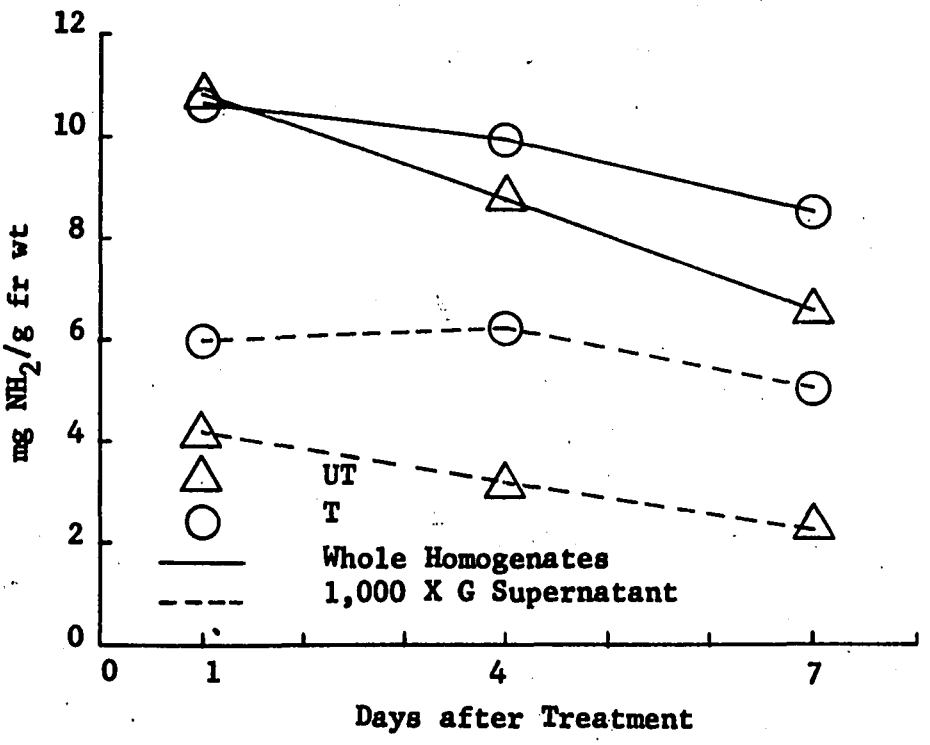


Figure 10. The effect of DIURON-DBSA on the amino nitrogen concentration of primary leaves.

due to an increase in leaf fresh weight with no change in leaf nitrogen content during this period.

The proportion of soluble nitrogen (that of the 1,000 X G supernatant) was higher in herbicide treated leaves (Figure 9). Increased soluble nitrogen has been associated with iron deficiency chlorosis. For example, Perur, et al. (1961) found a lower protein/N ratio for chlorotic corn leaves. The protein content of the chloroplast fraction was reduced 82 per cent and the reduction in total leaf protein was 25 per cent. By assuming that the difference between total amino nitrogen and supernatant nitrogen represents protein nitrogen in Figure 9, the reduction in protein nitrogen per leaf by herbicide treatment is 33 per cent by the seventh day.

Bingham and Porter (1961a) indicated that microscopic examination of cotton cotyledons showed that the rate and amount of cell enlargement was reduced by DCMA treatment. When slides from treated and control plants were compared, it became evident that the same was true with DIURON-DBSA treatment. In addition, the growth and development of vascular bundles was affected. Xylem vessels were smaller, fewer in number, and thinner walled for treated leaves (Figures 11, 12). Though less evident from photomicrographs, the phloem was also affected. Similar results have been reported for soybeans treated with monuron (Christoph and Fisk, 1954). In addition, they observed the collapse of the cambium and disorganization of the phloem. From the results observed here and those of Christoph and Fisk, it may be concluded that fewer conducting elements were present because of an effect on the cambium. Poor

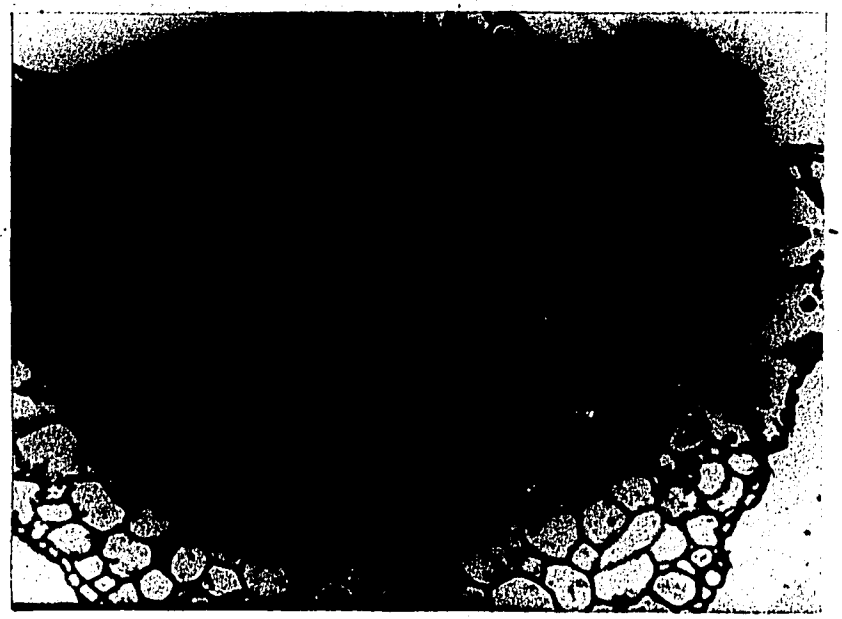


Figure 11. A photomicrograph showing the cross-section of an untreated primary leaf midrib (100 X magnification, (A) xylem vessel, (B) sieve cell of phloem.)

Figure 12. A photomicrograph showing the cross-section of a treated primary leaf midrib (100 X magnification, (A) xylem vessel, (B) sieve cell of phloem.)

differentiation of the elements may possibly be attributed to disruption of the process of differentiation. Reduction in photosynthetic activity should have had an effect on the stems, although it is possible that the stem sections examined were nearly mature at the time of herbicide application.

Apparently, the primary leaves intercepted most of the herbicide so that stem development below the primary leaves was little affected (Figures 5 and 6). The effect of the herbicide on the second internode (Figures 5, 6) may be regarded as strong evidence that the anatomy of this internode was affected by the herbicide treatment. The results of Christoph and Fisk (1954) showed that soybean stems were affected by monuron that was absorbed from the soil. Similar results are thought to be possible with diuron.

II. Effects of DIURON-DBSA on Leaf Respiration

It was decided to study the effect of the herbicide on the respiratory activity of soybeans. In the first experiment, soybeans were planted in the greenhouse and the herbicide was applied at the rate of one-fifth pound per acre. Respiration was determined on the second, fifth, and tenth days after herbicide treatment. Examination of Figure 13 shows that the herbicide treatment reduced both carbon dioxide evolution and oxygen consumption. Treated leaves showed a tendency to recover during the course of the experiment. It was at the completion of these experiments that the application rate of the herbicide was increased to three-eighths pound per acre. Study of respiratory quotients (RQ or

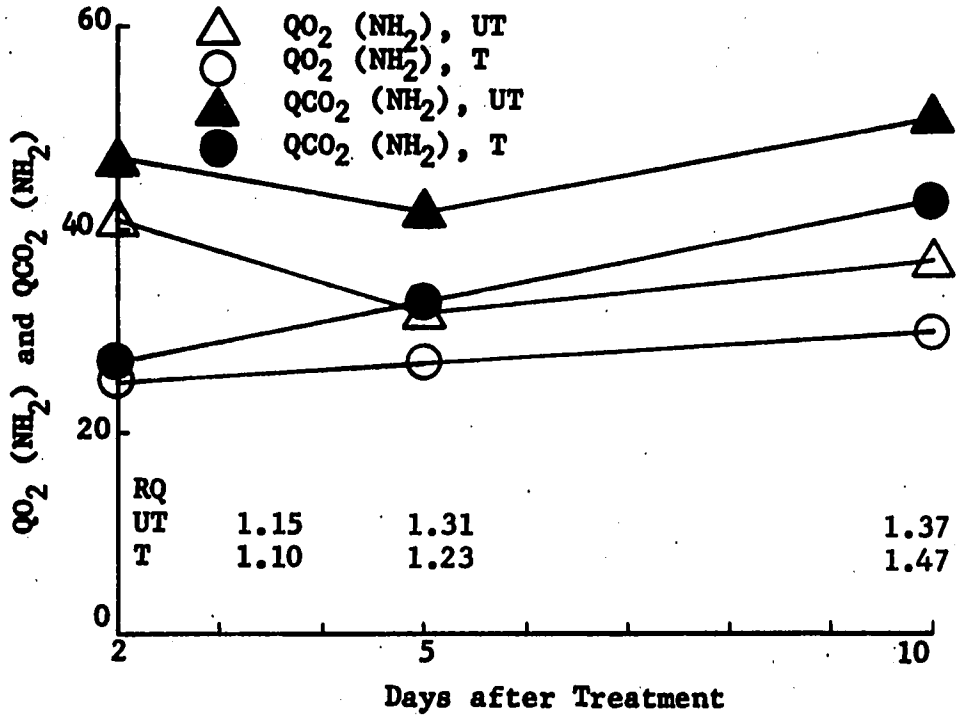


Figure 13. The effect of DIURON-DBSA on the respiratory activity of primary leaf disks.

ratio of volumes of CO_2 to O_2) indicate that there was a shift from carbohydrates to organic acids as the respiratory substrates during this period.

Some of the previously described experiments indicated that dark pre-treatment affected the response of the plants to DIURON-DBSA. It was decided to try to determine if the respiratory activity was also affected. Plants were grown in the greenhouse for six days and then moved to the growth room or darkroom. On the eighth day, one-half of the flats in each group were sprayed with the herbicide. Another experimental factor was added when plants were returned to the darkroom or growth chamber so that there were eight experimental combinations. Respiratory rates were determined at one and four days after herbicide treatment. The experiment was not continued beyond the fourth day because plants in the darkroom were becoming etiolated. The treatment combinations and results are graphically depicted in Figure 14.

The effect of a two-day dark period prior to application of the herbicide was to give a greater reduction in respiratory activity even though the plants were moved to the growth chamber after herbicide treatment. It may be noted that plants with light pre-treatment showed almost complete recovery by the fourth day with light after-treatment. With dark pre-treatment and dark after-treatment, there was continued decline in respiratory activity to the fourth day. Dark after-treatment, following any of the experimental combinations, resulted in reduced respiratory activity. Notable, however, is the tendency for the RQ values to rise with dark treatment following herbicide treatment.

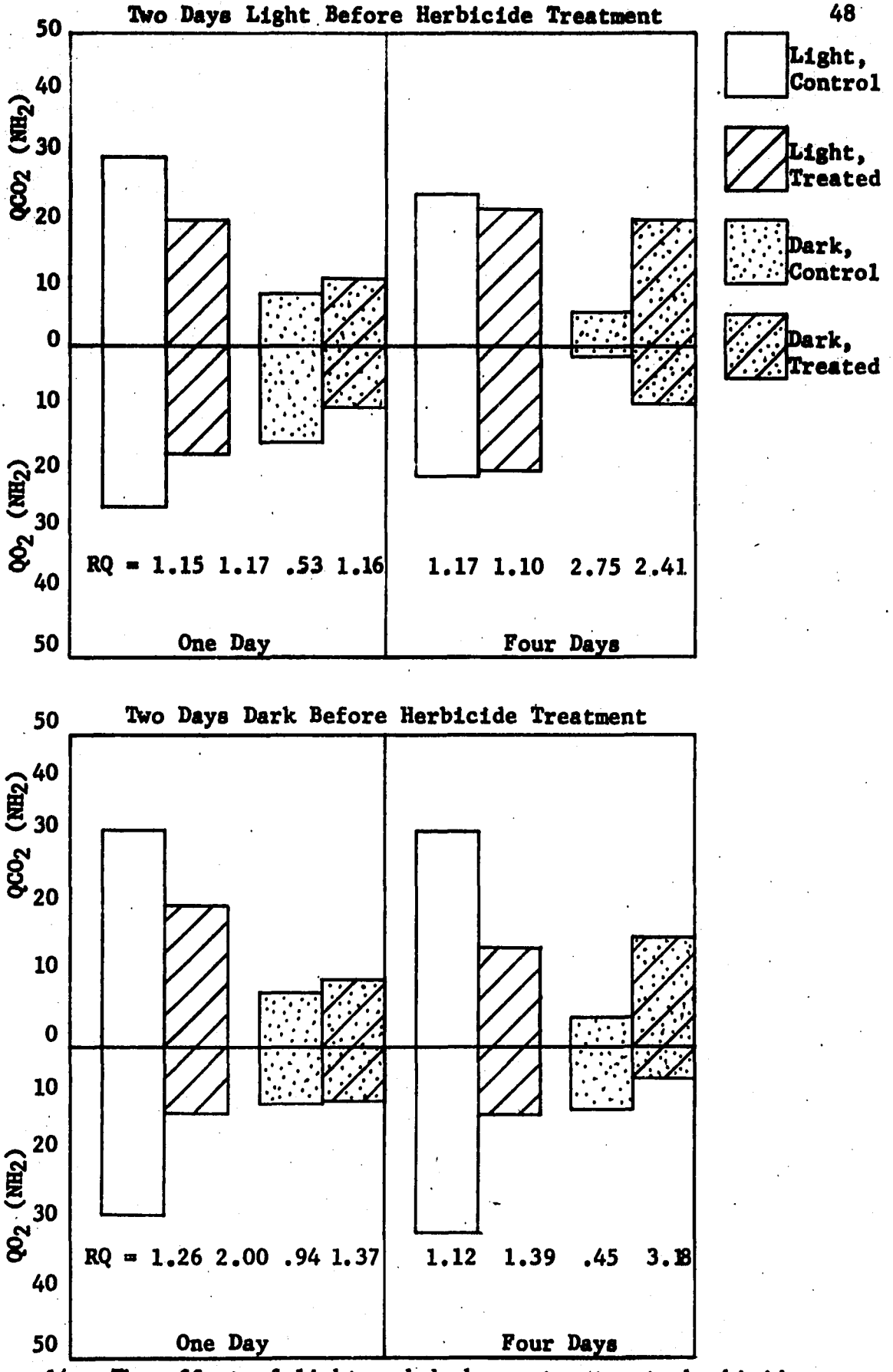


Figure 14. The effect of light and dark pre-treatment, herbicide treatment, and light and dark after treatment on the respiration of disks from soybean primary leaves.

The rise in RQ values could be intrepreted to mean that there was an increase in the proportion of respiration mediated by the Krebs cycle. It cannot be argued that the dark after-treatment resulted in the removal of a Krebs cycle inhibitor, because the untreated tissue did not show a similar response. An inhibition of the synthesis or metabolism of other substrates is a possibility.

It was previously noted that light treatment after herbicide treatment did not give good recovery of respiratory rates when the plants had a dark pre-treatment. This agrees with the opinion of Gentner and Hilton (1960) that the appearance of herbicide injury symptoms (physiological in this case) is related to the depletion of photosynthate. It would be interesting to make an anatomical study of soybeans that had diuron and sucrose to determine if anatomical abnormalities continued to occur.

III. Effects of DIURON-DBSA on Terminal Oxidases

A. Cytochrome Oxidase

Bingham and Porter (1961b) and Funderburk and Porter (1961) were unable to demonstrate the presence of cytochrome oxidase in cotton and corn. The success of Wu and Scheffer (1960) in demonstrating Krebs cycle activity in tomato leaves and stems indicated that positive results were possible with chlorophyllous tissue.

The Wu and Scheffer procedure was followed closely in the isolation of mitochondria from soybean stems. A pellet was found at the end of the procedure, which was suspended in 0.25 M sucrose. Some of the preparation was stained with Janus Green B (1/50,000) and observed under the oil-immersion lens of a microscope

(Millerd, et al., 1951). Numerous spheroids of varying size were observed. Perusal of several papers on this subject indicated that some might be mitochondria. However, other organelles of size and shape have been reported. The particles were kept in a deep-freeze until use.

Particulate preparations were made from a number of batches of soybeans. Attempts were made to determine enzyme activity by using succinate, malate, and pyruvate as substrates. The invariable result was either no change in manometer pressures or positive pressures. Addition of DPN (diphosphopyridine nucleotide), coenzyme A, cytochrome C, TPP (thiamine pyrophosphate), and ATP (adenosine triphosphate) did not change the results. Treatment of particles with bile salts was also ineffective.

It was apparent that some other approach would have to be considered. It was also rather evident that a gas which was not absorbed by the alkali produced the positive pressures. A double arm flask was used with alkali in the center well and concentrated sulfuric acid in one of the sidearms on the theory that the gas might react with the acid. However, this made no difference in the results.

Study of the literature indicated that oxygen could be produced by these preparations. This has been demonstrated by Daly and Brown (1954) by means of isotopic oxygen with a mass spectrometer. Other difficulties may have been encountered. There may, of course, have been little cytochrome oxidase in the stems. According to the inhibitor theory of DeAbramo, et al. (1958),

the Krebs cycle could have been inhibited by the combination of glyoxylate and oxaloacetate to form an inhibitor of aconitase. Another possibility is that the mitochondria were affected by the processes of isolation and storage. Results with particles from etiolated cotyledons indicated that freezing was injurious.

Howell (1961) was able to demonstrate Krebs cycle activity in particles from etiolated soybean cotyledons that were isolated by a procedure that was similar to that of Wu and Scheffer (1961). It was decided to try the same procedure that had been used for stems with etiolated cotyledons. Soybeans were germinated in a darkroom and particles were isolated on the ninth day. It was found that these particles oxidized succinate or succinate plus malate. Succinate was the most actively oxidized substrate (Figure 15). Positive pressures were found during the first 15 minutes, although a net oxygen uptake was found afterward. In this case, respiratory determinations were made immediately after isolating the particles. Activity of the particles was determined 15 days after being frozen and found to be considerably reduced. It should be noted that the actual amounts of oxygen consumed were small. The data in Figure 15 are ccnsiderably enlarged because they are based on one mg of amino nitrogen, and the nitrogen contents of particle preparations were low. The particles oxidized ascorbic acid at a rate that was approximately three times that for succinate.

After it had been demonstrated that active particle preparations could be prepared, it became of interest to determine if the

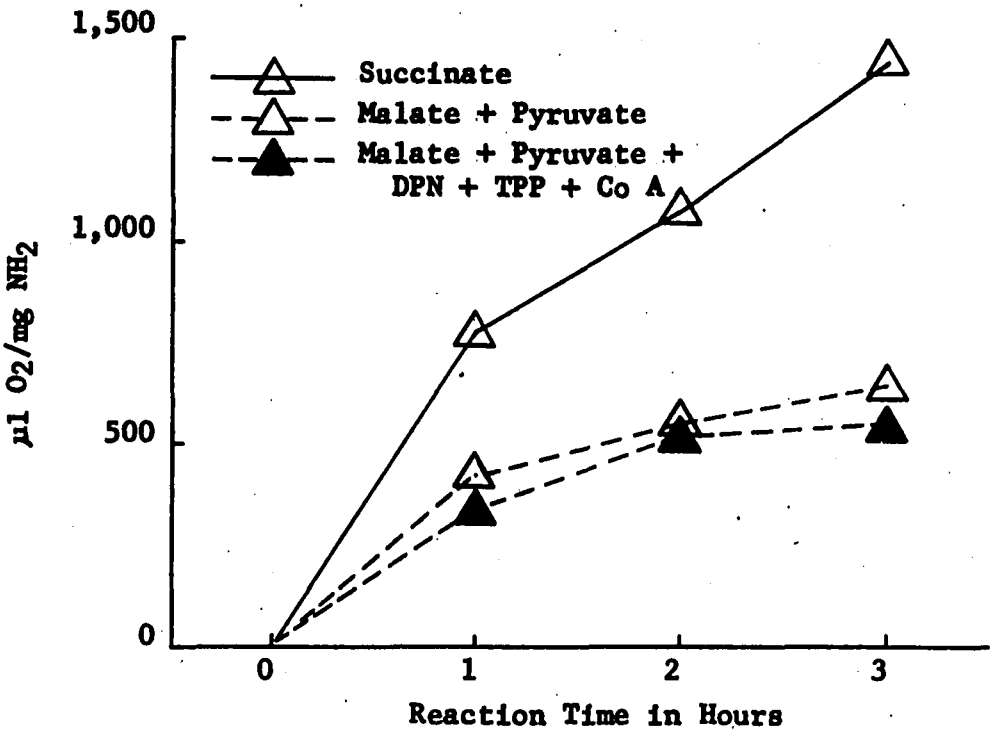


Figure 15. The oxidation of Krebs cycle substrates by "particles" from etiolated cotyledons.

herbicide had any effect on succinoxidase activity. There may be a question as to whether there is any practical importance in the effect of a herbicide on enzyme systems that could only be demonstrated in etiolated tissue. Residual diuron in the soil could conceivably affect the mobilization of stored foods (fats particularly) to a more useful form via the glyoxylate shunt in the cotyledons of germinating soybeans.

Soybeans were germinated in the dark with a control and herbicide treatment. The treatment consisted of applying 40 ml of 100 ppm diuron as DIURON-DBSA to one tray of beans. Determination of respiratory activity showed a reduction in succinoxidase activity with herbicide treatment (Figure 16). Boiling the enzyme preparations did not completely eliminate enzyme activity. As noted before, the actual oxygen consumption was low with the data having been expanded to the basis of one mg of amino nitrogen.

A logarithmic series of concentrations of potassium cyanide gave increasing inhibition with increasing concentration of the inhibitor. One mM of cyanide gave 69 per cent inhibition (Figure 17). This concentration is commonly used for the inhibition of cytochrome oxidase (James, (1953). When cyanide was added, the activity of the treated preparations was noticeably inferior to the activity of untreated preparations.

B. Ascorbic Acid Oxidase

The first of these various studies concerned ascorbic acid oxidase activity. DCMA at the rate of three pounds per acre was used as the herbicide at that time. The soybeans were usually

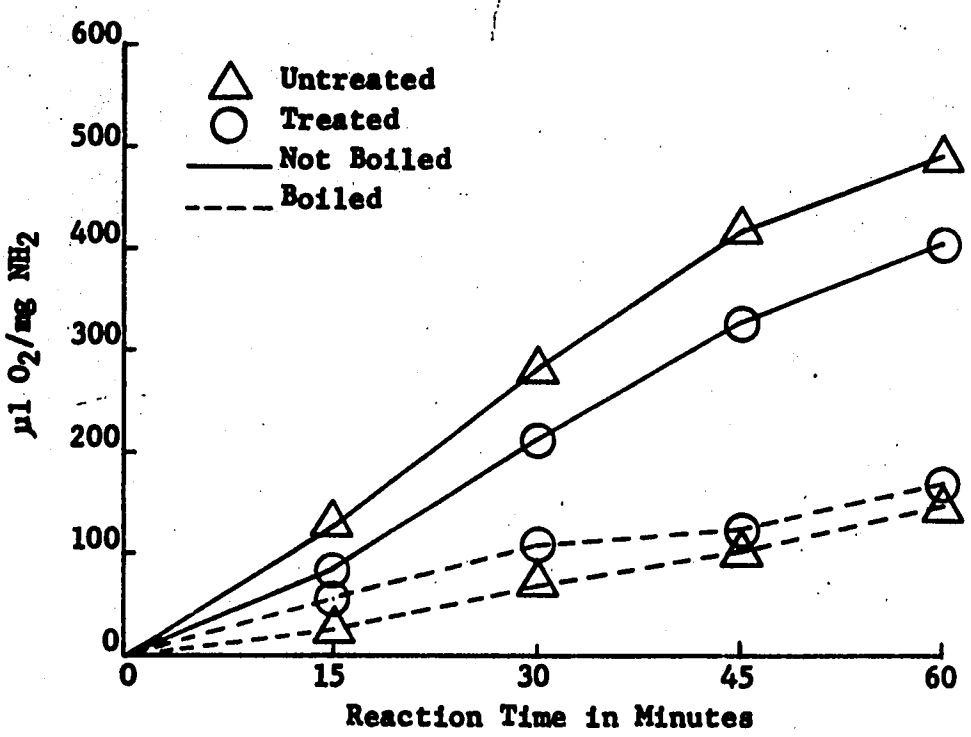


Figure 16. The effect of DIURON-DBSA on the oxidation of succinate by "particles" from etiolated cotyledons.

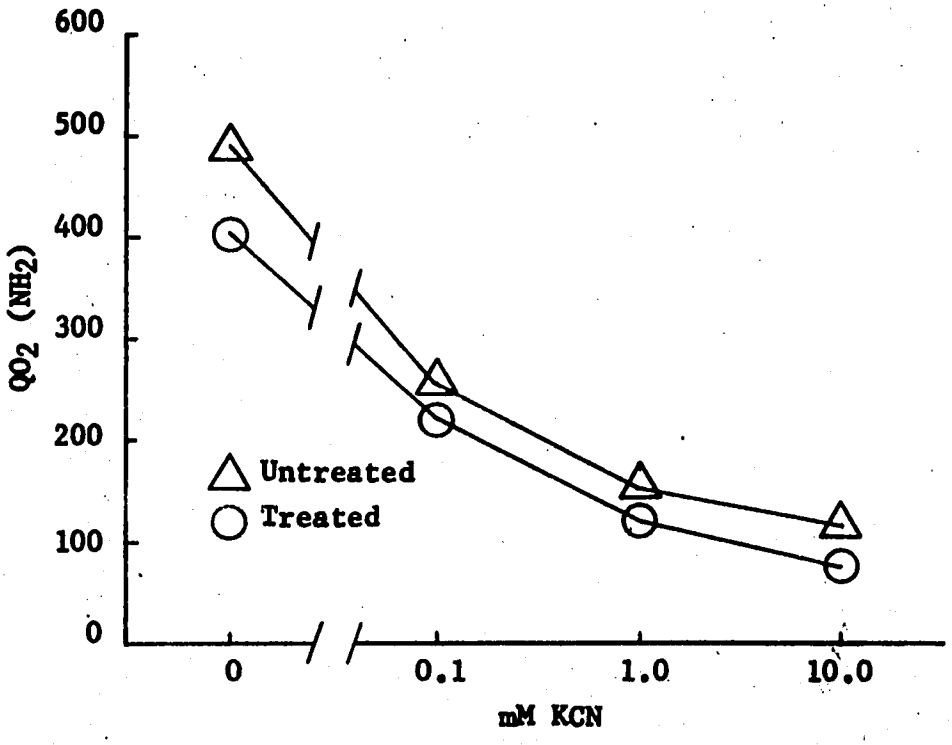


Figure 17. The effect of potassium cyanide on the oxidation of succinate by "particles" from etiolated cotyledons.

treated on the sixth day after planting, but no particular schedule was followed for studying the effects of the herbicide on the plants.

One early study was concerned with the effect of pH on the ascorbate activity of whole homogenates from leaves. Homogenates were made with phosphate buffer (0.05 M) with pH values from 4 to 8. It can be noted in Figure 18 that the effect of pH on the ascorbate activity of the homogenates was slight between pH 5 and 7. Homogenates from DCMA treated plants were usually slightly less active than control homogenates in the oxidation of ascorbate.

Homogenates were boiled to determine if the oxidation of homogenates was enzymatic in nature. Boiling was very effective in preventing the oxidation of ascorbic acid, which indicates that the process is enzyme catalyzed (Figure 19). It may be noted that positive pressures were found with boiled homogenates. In fact, this almost always occurred with boiled homogenates. The nature of the gas causing this pressure is unknown.

Homogenates were centrifuged at 1,000 X G, and the supernatants were decanted. Phosphate buffer was added to the sediment and to the supernatant to give the original volume of whole homogenate. Only positive pressures were found for the supernatants. The cell wall fraction actively oxidized ascorbic acid, and this activity could be destroyed by boiling. These results agree with those of Bingham and Porter (1961b) for cotton in that the ascorbic acid oxidase was found in the cell wall fraction and was enzymatic in nature. However, Bingham and Porter found that DCMA reduced

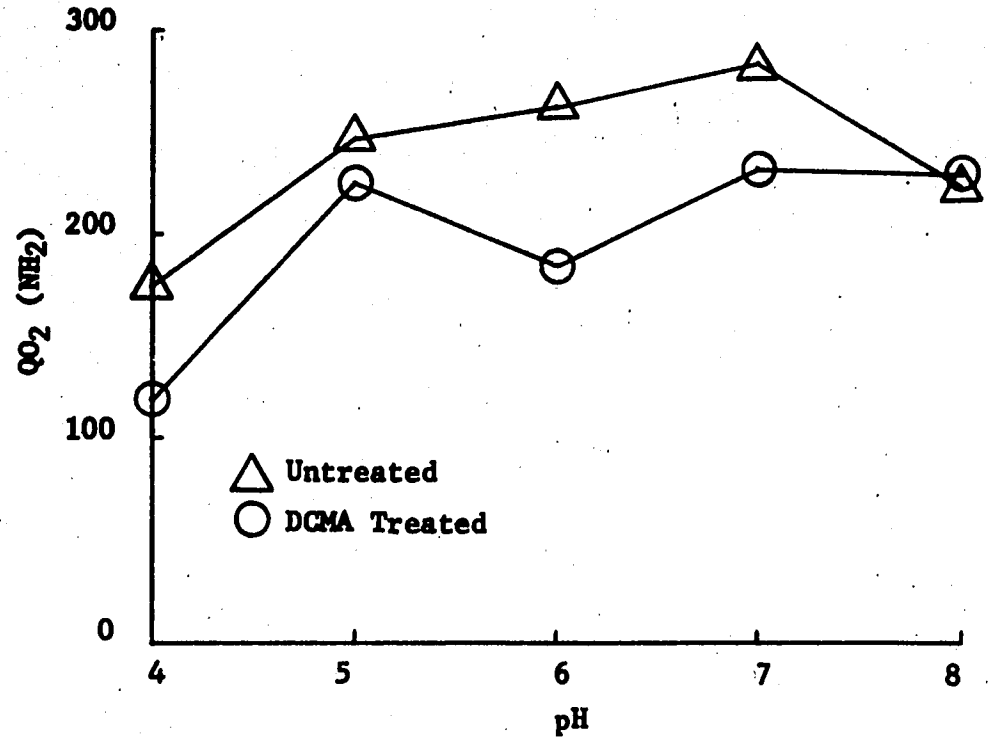


Figure 18. The effect of pH on the oxidation of ascorbate by whole homogenates from primary leaves.

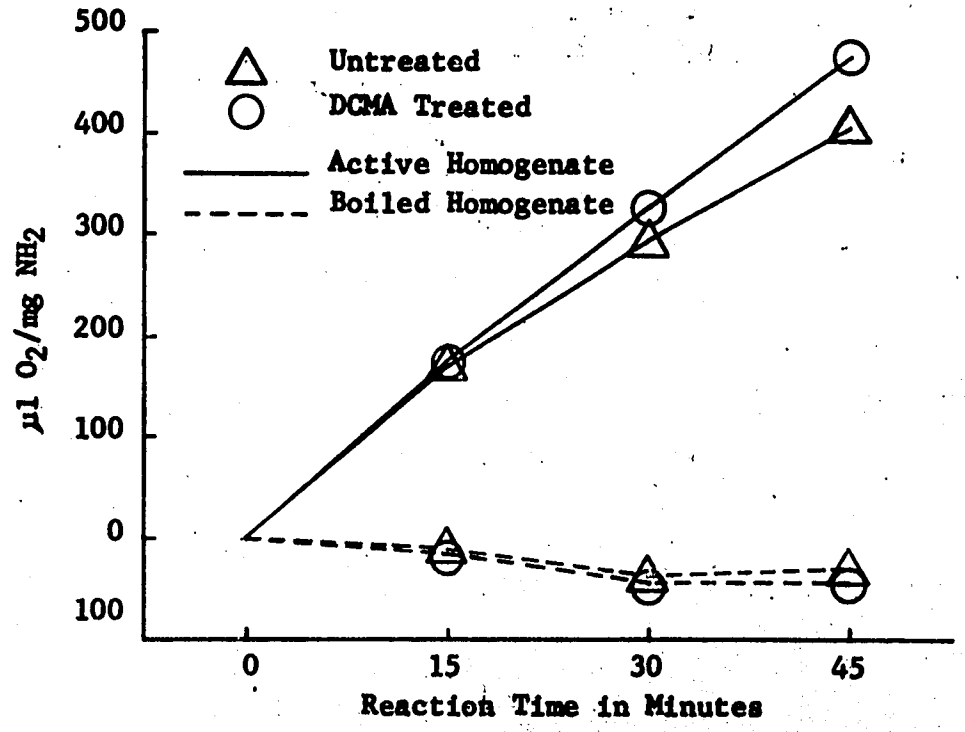


Figure 19. The effect of boiling on the oxidation of ascorbate by whole homogenates from primary leaves.

ascorbic acid oxidase activity to the same degree that growth was reduced. It is possible, of course, that ascorbic acid oxidase activity could have been affected if the herbicide had been applied at an earlier stage. The oxidation of ascorbic acid by corn homogenates was found to be non-enzymatic and was stimulated by DCMA treatment (Funderburk and Porter, 1961).

The ascorbic acid oxidase activity of soybean was further studied after DIURON-DBSA was selected as the experimental herbicide. The ascorbic acid oxidase activities of control and treated leaves and cotyledons are presented in Figure 20. These results were obtained one day after herbicide treatment or nine days after the start of germination. It can be seen that the ascorbic acid oxidase activities of the leaves were greater than for cotyledons. Study of Figure 21 indicates that the relative differences were greater when the results were expressed on a per leaf or per cotyledon basis. This is due to the cotyledons having higher amino nitrogen contents than the leaves. The ascorbic acid oxidase activity of cotyledons was not followed for a period of several days because the cotyledons were falling from the plants by 12 days after planting or four days after herbicide treatment. Study of Figures 20 and 21 indicates that there was little difference in the ascorbic acid oxidase activities of treated and control tissue. This is further supported by the results presented in Figure 22, where the trend in ascorbic acid oxidase was followed for one weed after herbicide treatment.

The effect of 0.2 mM DDC (diethyldithiocarbamate) in inhibiting the activity of ascorbic acid oxidase is depicted in

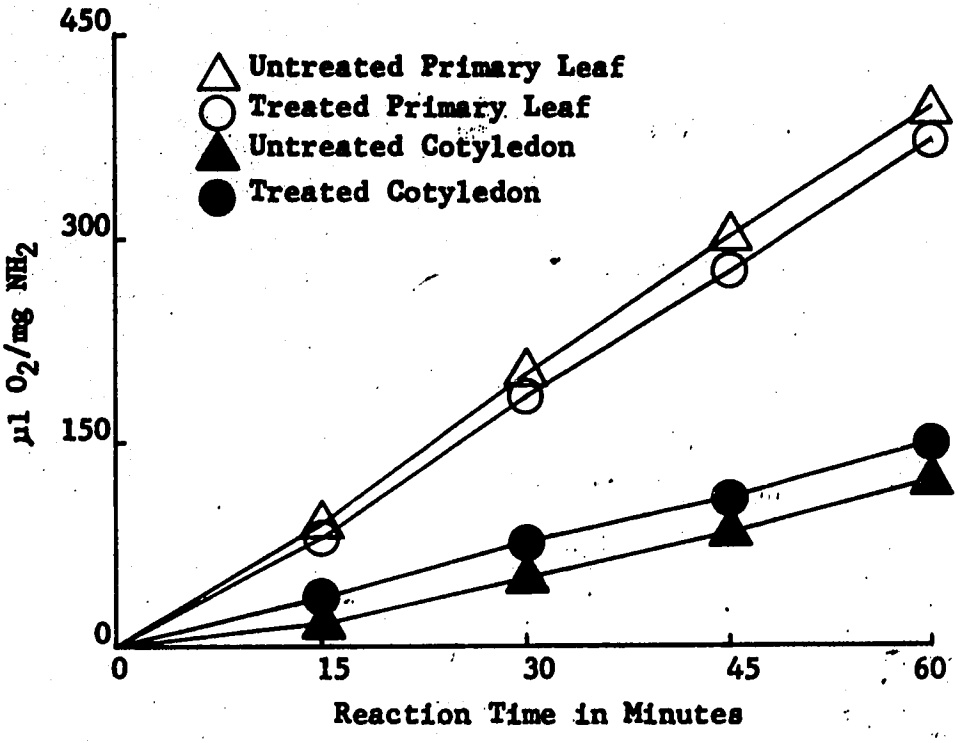


Figure 20. The oxidation of ascorbate (per mg N) by whole homogenates from primary leaves and cotyledons one day after DIURON-DBSA treatment.

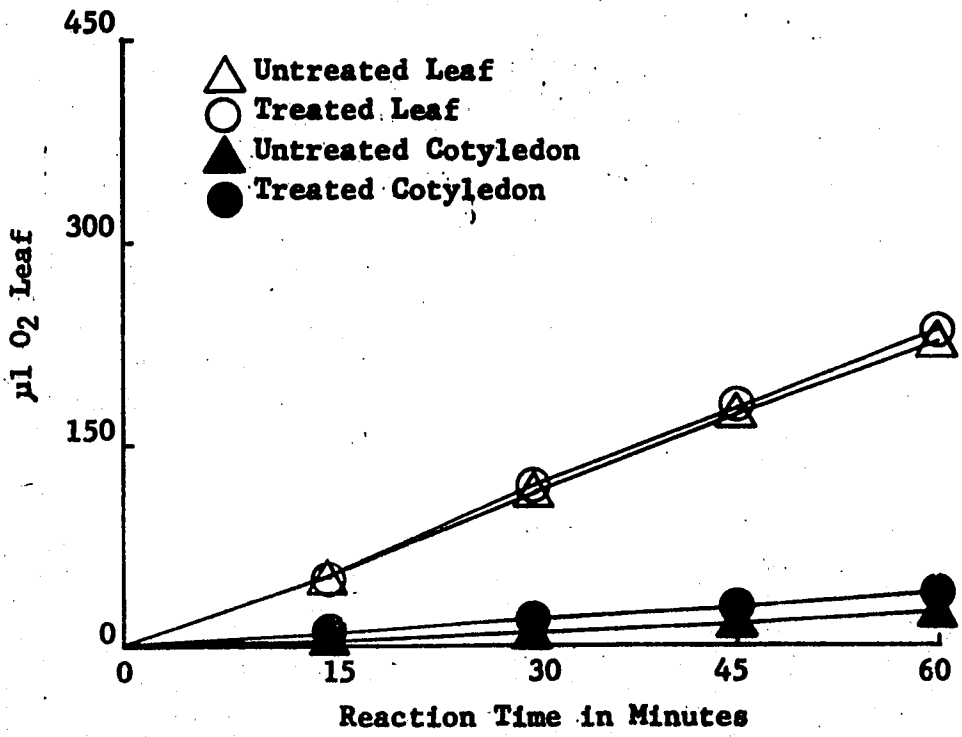


Figure 21. The rate of oxidation of ascorbate (per leaf) by homogenates from primary leaves and cotyledons one day after DIURON-DBSA treatment.

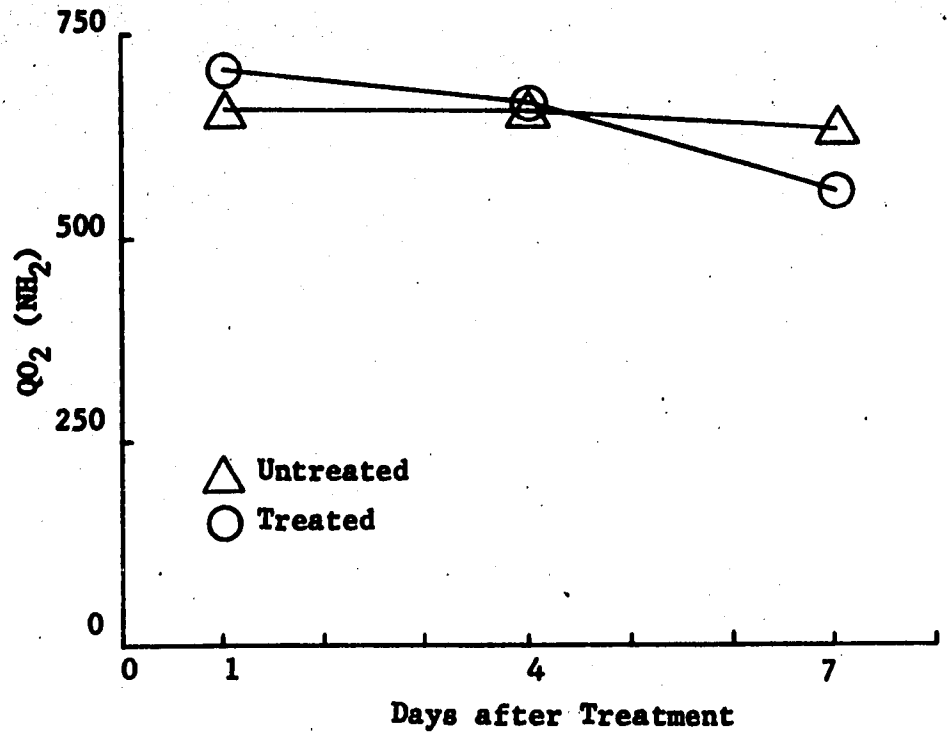


Figure 22. The effect of DIURON-DBSA on ascorbic acid oxidase activity of soybean primary leaves.

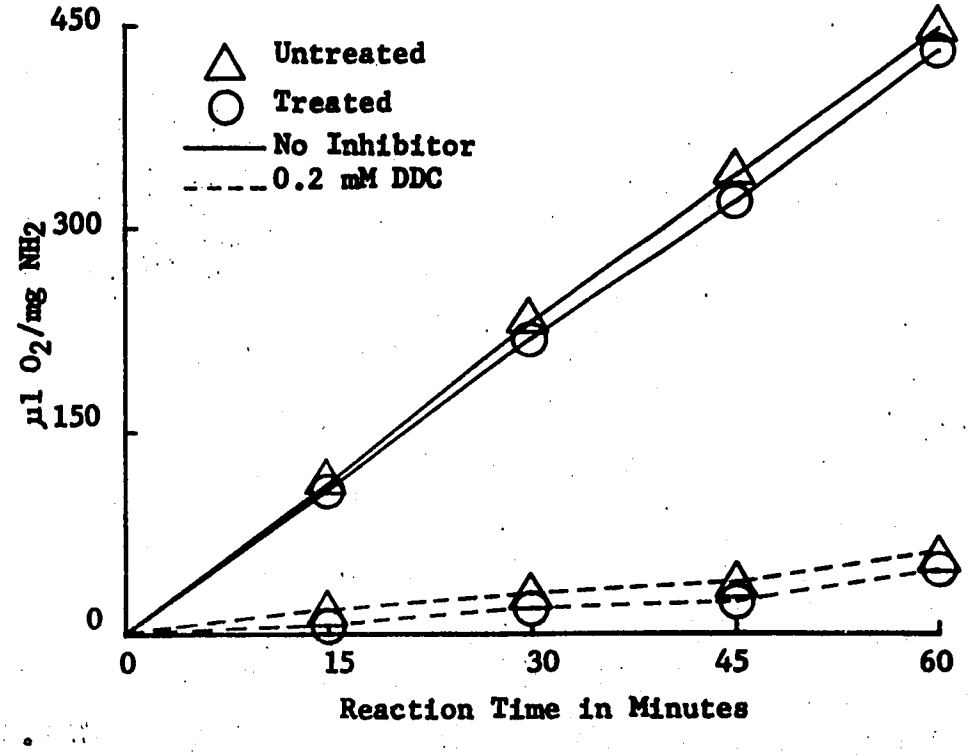


Figure 23. The inhibition of ascorbic acid oxidase by 0.2 mM DDC (Diethyldithiocarbamate).

Figure 23. DDC inhibits ascorbic acid oxidase at 0.2 mM without having any great effect on cytochrome oxidase according to James (1953). In other experiments, 0.2 mM 8-hydroxyquinoline was found to effectively inhibit ascorbic acid oxidase.

Observation indicated that ascorbic acid oxidase activity was less in plants grown from older seed. Activity was very high in plants grown from seed that had been recently harvested as depicted in Figure 22. At the same time, germination of seed and growth of seedlings were prompt, vigorous, and uniform. These observations were never systematically investigated; however, such a relationship seems possible in view of the idea that cell wall growth is related to ascorbic acid oxidase activity (Mertz, 1961a; Newcomb, 1960).

C. Polyphenol Oxidase

The phenol oxidases are a group of enzymes that catalyze the oxidation of mono-phenols and ortho-diphenols. They are commonly found in monocots and dicots (Boswell and Whiting, 1938). They seem to be particularly active in tubers such as those of the potato and nutsedge (Baker and Nelson, 1943; and Palmer and Porter, 1959b). The activity of the phenol oxidases is often linked to darkening of tissues such as that of bruised or damaged potatoes (Robinson and Nelson, 1944). Sisler and Evans (1958b) demonstrated that polyphenol oxidase could accept electrons from reduced pyridine nucleotides and function as a terminal oxidase.

Study of polyphenol oxidase activity was begun after DIURON-DBSA was adopted as the experimental herbicide. Some early

experiments involved soybeans that were treated with the herbicide at the rate of one-fifth pound per acre. The treated leaves were often only slightly affected and appeared to recover from the herbicide treatment.

Catechol was the only substrate that was used for polyphenol oxidase activity. Boiling of homogenates was found to destroy the oxidation of catechol or to produce positive pressures with simple leaf homogenates. It was found that homogenates of cotyledons showed only positive pressures. The polyphenol oxidase activity of simple leaves was low as the oxygen consumption seldom exceeded 50 μl O_2/ mg N/ hr. This was approximately the same as that found by Bingham and Porter (1961b) for cotton cotyledons. However Sunderburk and Porter (1961) found that the polyphenol oxidase of corn was considerably more active than this.

The polyphenol oxidase activity for simple leaves on the ninth day after herbicide treatment is given in Figure 24. The herbicide treatment reduced the enzyme activity by approximately one-half. DDC at 0.2 mM gave relatively better inhibition for treated samples than for the untreated. Two-tenths mM 8-hydroxyquinoline was found to give better inhibition with homogenates from control tissue than 0.2 mM DDC. Both compounds are considered to be effective inhibitors of polyphenol oxidase at 0.2 mM (James, 1953; and James and Garton, 1952). When the data for this experiment were based on a gram of fresh tissue, the relative enzyme activities of the treatments were not affected very much (Figure 25).

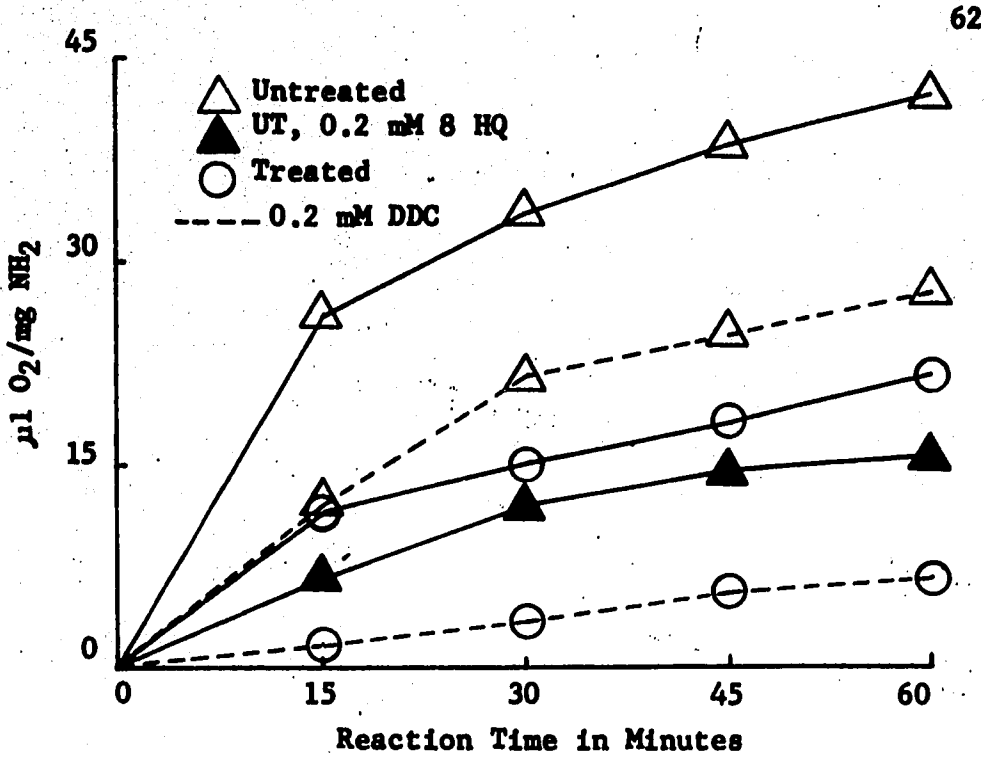


Figure 24. The effect of DIURON-DBSA applied at the rate of one-fourth pound per acre on polyphenol oxidase activity per mg nitrogen.

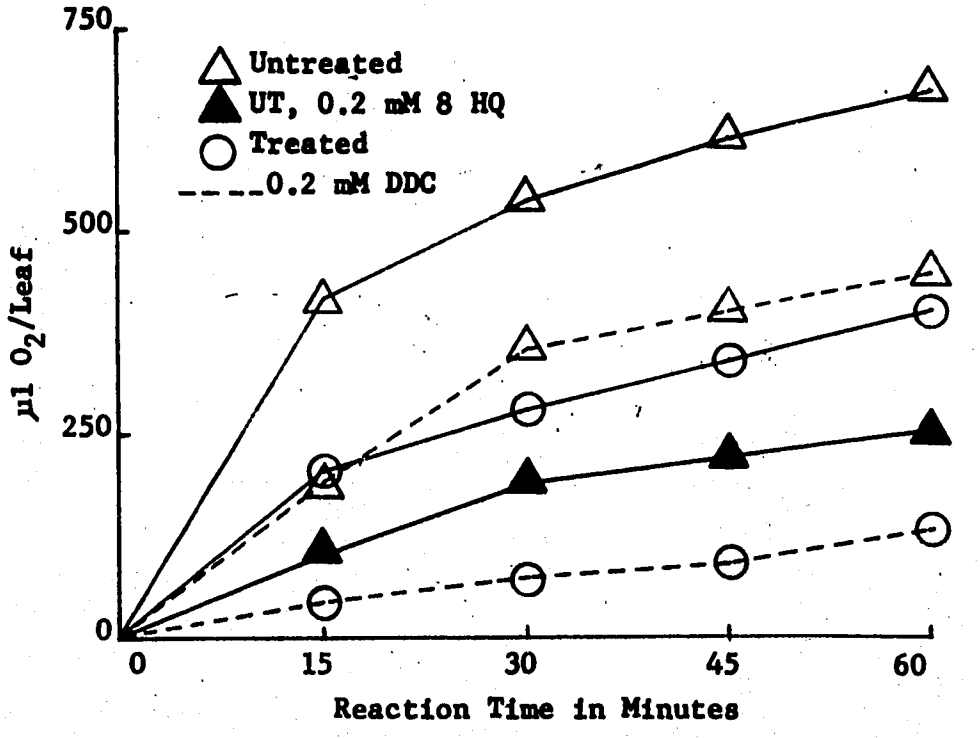


Figure 25. The effect of DIURON-DBSA applied at the rate of one-fourth pound per acre on polyphenol oxidase activity per gram fresh weight of primary leaf.

The polyphenol oxidase activity of simple leaves was followed for a seven day period after spraying at the rate of three-eighths pound of DIURON-DBSA per acre. It can be seen in Figure 26 that enzyme activity increased with leaf age. Enzyme activity was unaffected until after the fourth day when the activity of homogenates from herbicide-treated plants became stationary. Polyphenol oxidase activity rates were always small in comparison with other kinds of enzymatic activity.

DDC did not give very effective inhibition of polyphenol oxidase activity as can be discerned from Figure 27. Again, 0.2 mM DDC gave better inhibition of the polyphenol oxidase activity of homogenates from treated leaves than from the untreated.

D. Catalase

Catalase is an enzyme of universal occurrence in living cells. It is a metallo-protein with iron-hematin as its prosthetic group, and it has the specialized function of catalyzing the breakdown of hydrogen peroxide to water and oxygen. Hydrogen peroxide is a product of oxidations that are catalyzed by the flavoprotein enzymes, which will be discussed further in connection with glycolic acid oxidase.

Catalase activity was followed for one week after treatment with DIURON-DBSA at the rate of three-eighths pound per acre. Catalase activity was found to be very high as the maximum production of oxygen was in excess of 5,000 μl O_2/mg N/ 10 min. There was a notable tendency for catalase activity to increase with leaf age (Figure 28).

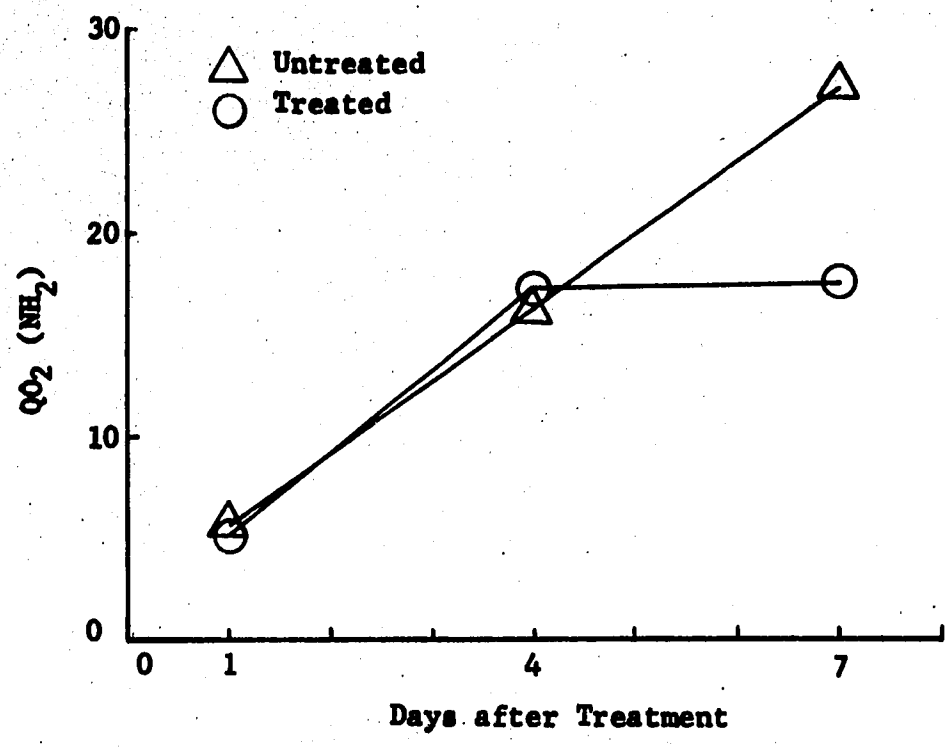


Figure 26. The effect of DIURON-DBSA on the polyphenol oxidase activity of soybean primary leaf homogenates during the testing period.

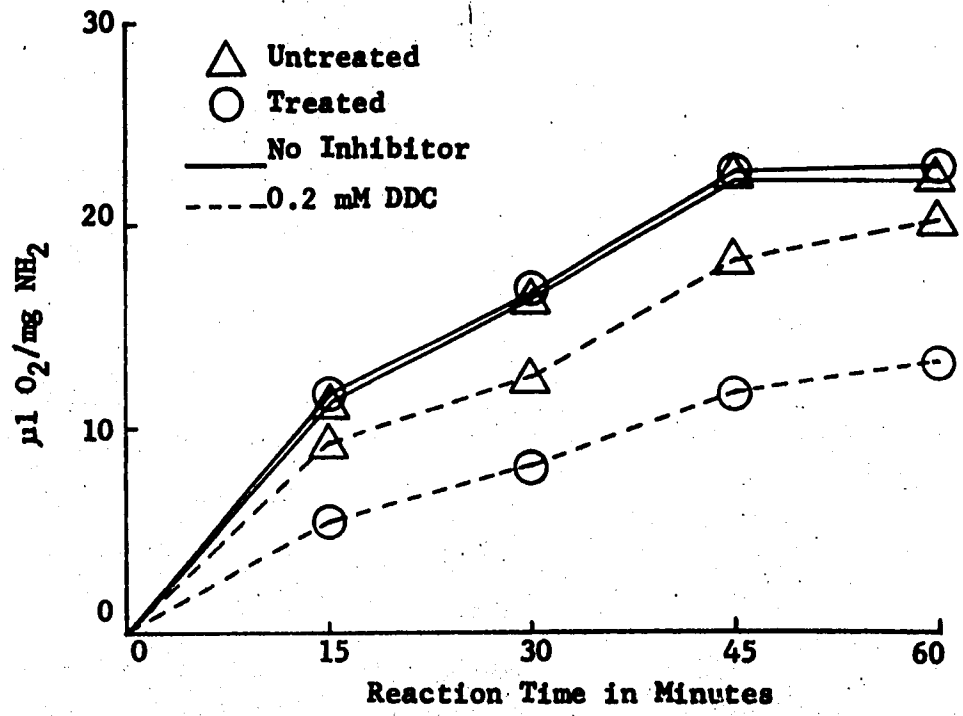


Figure 27. The inhibition of polyphenol oxidase by 0.2 mM DDC four days after herbicide treatment.

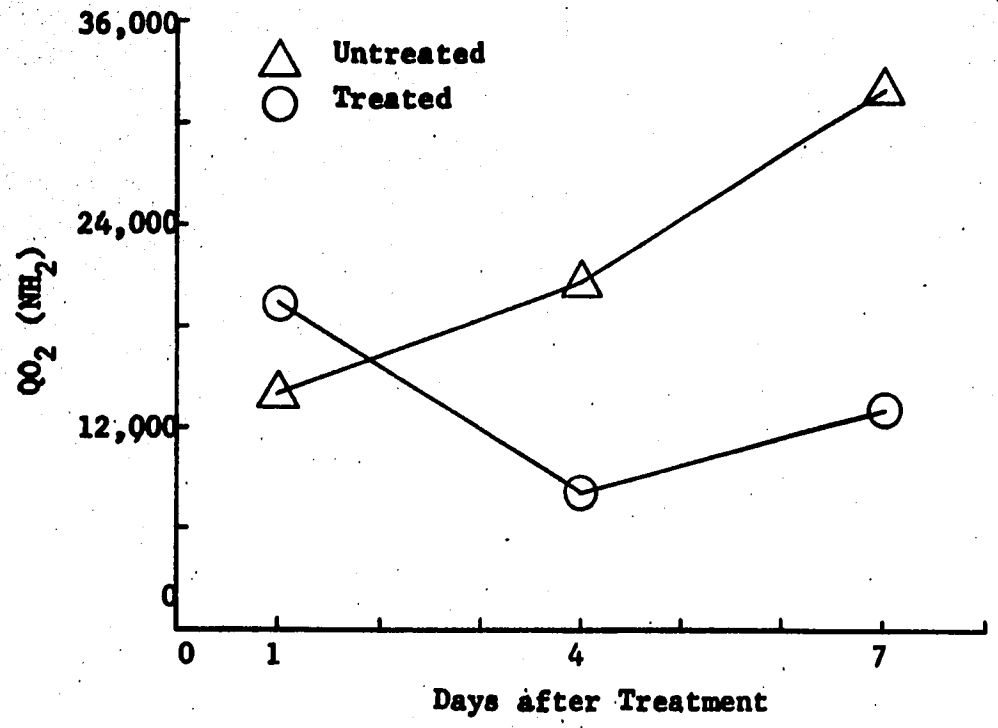


Figure 28. The effect of DIURON-DBSA on the catalase activity of 1,000 X G supernatants of soybean primary leaves.

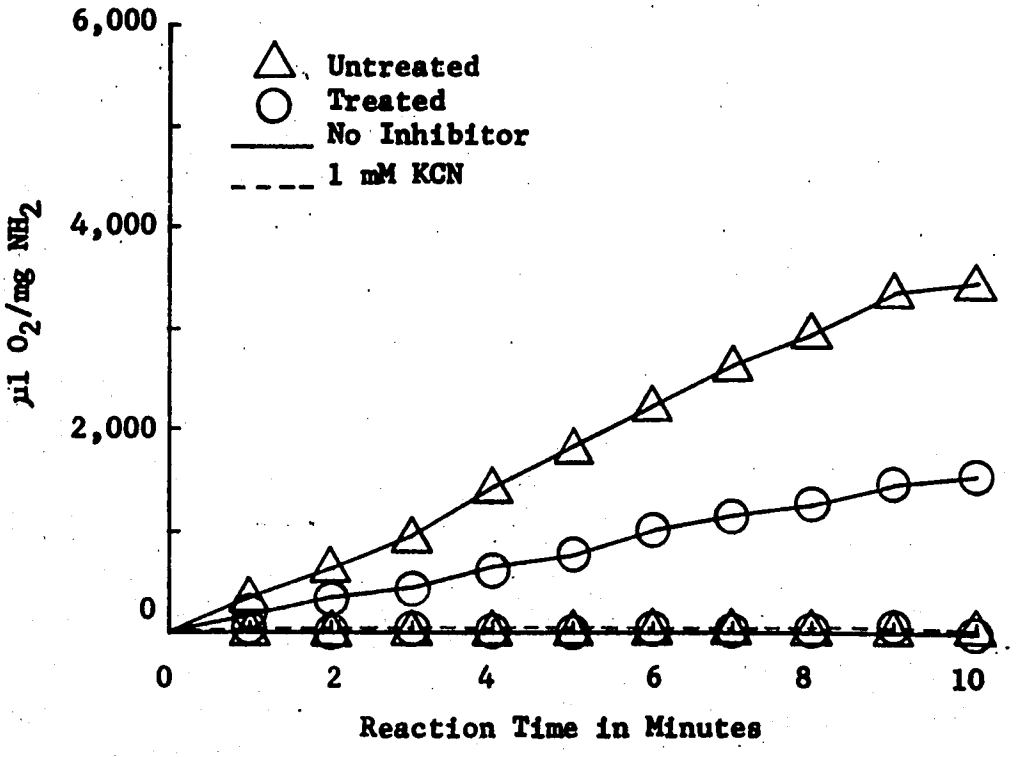


Figure 29. The effect of one mM KCN on catalase activity four days after herbicide treatment.

Catalase activity was almost completely inhibited by treatment with one mM potassium cyanide (Figure 29). Cyanide forms complexes with the iron of plants and effectively inhibits enzymes that have iron in their prosthetic groups.

Catalase activity was determined using the 1,000 X G supernatants of leaf homogenates in which there was increased soluble nitrogen due to herbicide treatment. It was apparent that this would have some effect on the results, and the data were converted to the basis of fresh weight (Figure 30). With one gram of fresh weight as the base, there was little change in catalase activity of untreated supernatants. On the other hand, there was decreased activity for the treated supernatants on the fourth and seventh days as found for catalase activity based on nitrogen. If the data were converted to that per leaf, there would have been an increase in catalase activity during the seven day period, because leaf weights increased during this period.

If fresh weight or nitrogen are used as the base, the herbicide treatment sharply reduces catalase activity on the fourth and seventh days. Funderburk and Porter (1961) reported a similar effect for DCMA on the catalase activity of corn. Bingham and Porter (1961c) reported that the catalase activity of DCMA-treated cotton cotyledons remained unchanged while that of the untreated cotyledons increased with age of the plant.

A number of chemicals reportedly reduce the catalase activity of plants. Especially notable is amino triazole (Palmer and Porter, 1959c; Pyfrom, et al., 1957; and Heim, et al., 1956).

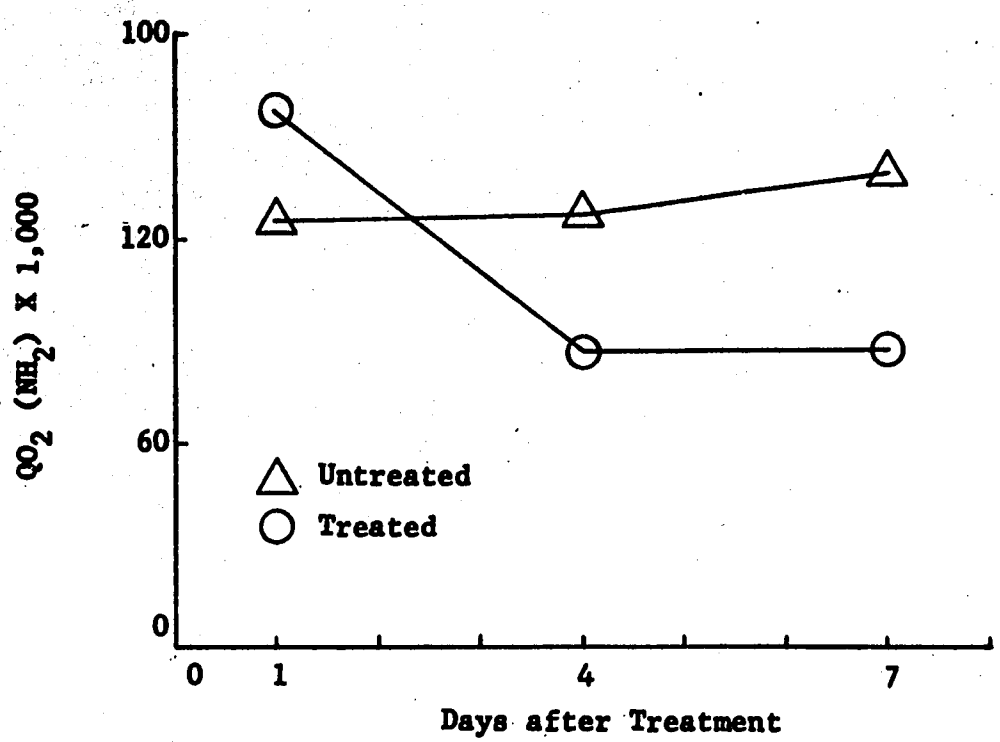


Figure 30. The effect of DIURON-DBSA on catalase activity per gram fresh weight.

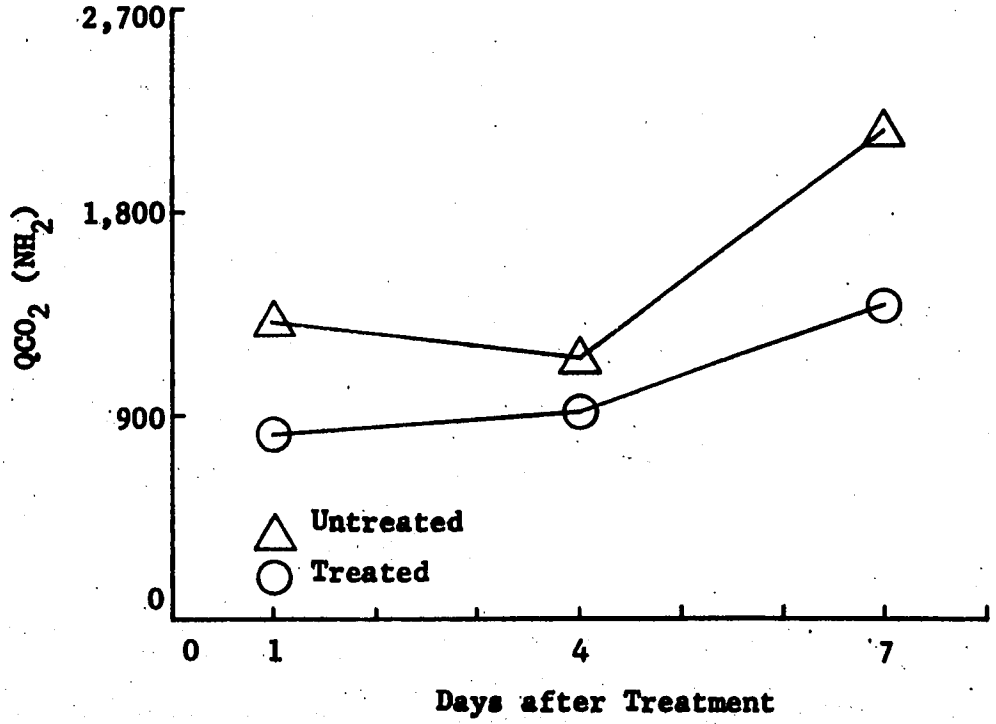


Figure 31. The effect of DIURON-DBSA on the peroxidase activity of 1,000 X G supernatants of soybean primary leaves.

Landon reported that the catalase activity of plants was reduced by treatment with ammonium sulfocyanate, thiourea, ethylene oxide, diethylene oxide, propylene oxide, sodium arsenite, or sodium chlorate. Reduced catalase activity has been reported for green or albino plant tissue with simple or manganese induced iron deficiency (Weinstein and Robbins, 1955).

It is interesting to speculate about the cause for the reduction in catalase activity of the DIURON-DBSA treated tissue. Herbicide treatment could lead to a reduction in the synthesis of the protein fraction of the enzyme as evidenced by increased soluble nitrogen in the treated tissue.

The formation of the iron-hematin complex or the union of the complex with protein could be affected. On the other hand, the normal activity of the enzyme could be inhibited by the herbicide. The fact that catalase can be affected by a number of chemicals indicates that its formation or activity is rather easily disturbed. The effect of amino triazole on rat liver catalase activity indicates that catalase can be affected independently of the photosynthetic process of plants. The effect of diuron and related herbicides on the Hill reaction is, of course, well known (Gentner and Hilton, 1960).

However, simple or induced iron deficiency can cause reduced catalase or cytochrome oxidase activity in green or albino plant tissues. At the same time, protein nitrogen is reduced in green or albino tissue by simple iron deficiency (Weinstein and Robbins, 1955). Iron deficiency or excess manganese is thought to reduce

the competitive advantage of iron for combining with the heme nucleus. The various chemicals that affect catalase may have the same effect. It should be noted that the activities of peroxidase and cytochrome oxidase were likewise reduced by DIURON-DBSA treatment. These enzymes, of course, have iron-hematin prosthetic groups.

In addition, iron has long been recognized as the element that is most difficult to supply to plants in an available form. It would be interesting to know if the herbicide affected the iron concentration or contents of leaves. It has been noted previously that the herbicide affected the development of conduction tissue, which could conceivably produce iron deficiency. If that were the case, an explanation would be evident for a number of the effects of this herbicide.

E. Peroxidase

Peroxidase is a commonly occurring enzyme that catalyzes the oxidation of phenols and aromatic amines (Lardy, 1950). The peroxidase activity of soybean primary leaves was considerably less than that reported by Bingham and Porter (1961c) for cotton leaves and cotyledons. Perusal of Figure 31 indicates that the herbicide treatment reduced peroxidase activity with nitrogen as the base. As had been found in other experiments, the nitrogen concentration in treated leaves decreased less than in the untreated leaves during this period. The use of nitrogen as the base exaggerated the differences between treated and untreated preparations as can be ascertained in Figure 32 with fresh weight as the

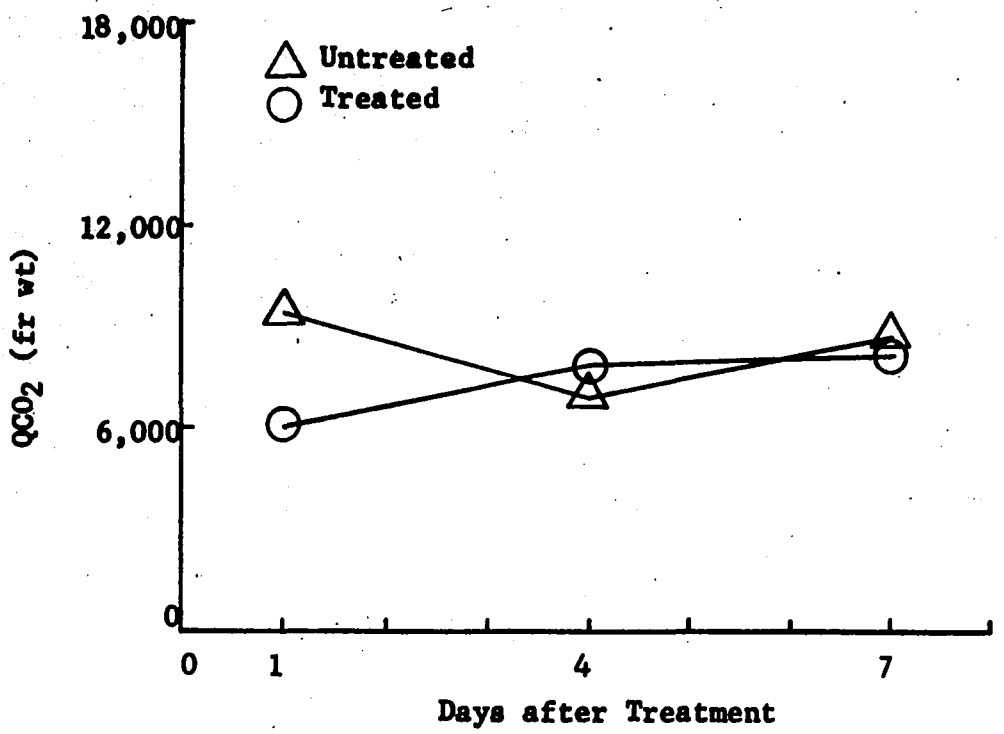


Figure 32. The effect of DIURON-DBSA on the peroxidase activity of 1,000 X G supernatants based on fresh weight.

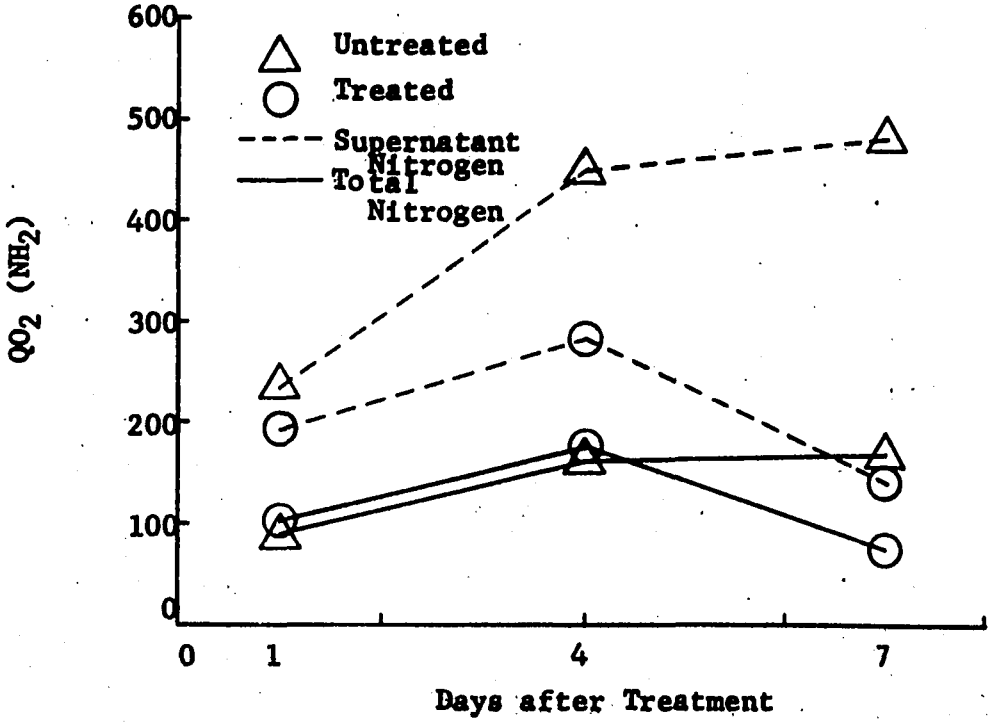


Figure 33. The effect of DIURON-DBSA on the oxidation of glycolic acid by 1,000 X G supernatants.

base. Peroxidase activity would be higher on a per leaf basis for the untreated preparations, because leaf weight increases at a faster rate for untreated plants during this period. Actual peroxidase activity was low, and the activities shown in Figures 31 and 32 were greatly expanded during the conversion to nitrogen or fresh weight bases. Cyanide was an effective inhibitor of peroxidase activity.

Other chemicals have been reported to affect the peroxidase activity of plant preparations. DCMA prevented an increase in the peroxidase activity of cotton leaves and cotyledons while that of the untreated plants increased with leaf age (Bingham and Porter, 1961c). Funderburk and Porter (1961) reported that DCMA reduced the peroxidase activity of corn leaves. Palmer and Porter (1959c) reported that amitrol (AT) maintained the peroxidase activity of "nut grass tubers" at the dormant level, while that of germinating untreated tubers increased 2.5 times. Heim, et al. (1956) reported that amitrol temporarily reduced the peroxidase activity of rat liver preparations.

Peroxidase has iron-hematin as its prosthetic group. It is thought that the herbicide is likely to affect the activity of peroxidase as it does the activity of catalase.

F. Glycolic Acid Oxidase

Glycolic acid oxidase is a flavoprotein enzyme that has riboflavin monophosphate for its prosthetic group (Frigerio and Harberry, 1958). This enzyme was originally thought to be absent in etiolated plants (Claggett, et al., 1949). Later work has shown

that the enzyme does occur in etiolated plants, but its activity is greatly enhanced by illumination (Noll and Burris, 1954). Glycolic acid is the natural substrate for glycolic acid oxidase; however, the oxidation of lactic acid is also catalyzed by glycolic acid oxidase (Zelich and Ochoa, 1953).

Zelich (1953) suggested that pyridine nucleotides, glyoxylic acid reductase, and glycolic acid oxidase could function as a hydrogen transferring system in the respiration of green leaves. The system would involve the reduction of glyoxylic acid to glycolic acid by means of reduced pyridine nucleotides and glyoxylic acid reductase. The oxidation of glycolic acid to glyoxylic acid by means of glycolic acid oxidase would complete the system by the production of hydrogen peroxide. The activity of the enzyme catalase would prevent the accumulation of hydrogen peroxide in plant tissues.

The difficulty in demonstrating the presence of cytochrome oxidase and Krebs cycle activity in photosynthetic tissue has made this idea credible. For example, Daly and Brown (1954) reported that the carbon monoxide sensitivity of leaves decreased with increase in leaf age. Various α-hydroxysulfonates have been found to be effective inhibitors of glycolic acid oxidase (Zelich, 1957). Studies with inhibitors of this type have indicated that as much as one-half of the carbon that is fixed by leaves in photosynthesis may be metabolized by this enzyme (Zelich, 1958; 1959). Also, D'Abramo, et al. (1958) postulated that glyoxylate combines with oxaloacetate to form an inhibitor of the Krebs cycle in leaves.

This idea is, of course, compatible with the observation that leaf sensitivity to carbon monoxide decreases with increasing leaf age (Daly and Brown, 1954). Further, Zelich and Barber (1960) reported that Krebs cycle activity decreased with leaf age.

The glycolic acid oxidase activity of 1,000 X G supernatants was studied over a seven day period following herbicide treatment. Glycolic acid oxidase activity was found to be comparable with ascorbic acid oxidase activity, when nitrogen was used as the base of comparison (Figures 22 and 33). Glycolic oxidase activity increased with leaf age as has been reported by Zelich (1957). The herbicide treatment resulted in smaller increases in enzymatic activity from the first to the fourth day after herbicide treatment. The glycolic acid oxidase activity of treated supernatants exhibited a sharp decrease by the seventh day. When enzyme activity was based on the nitrogen contents of whole homogenates, similar trends were noted, although activity was less per mg of amino nitrogen. Decreased enzyme activity for the treated preparations was usually found by the seventh day after herbicide treatment.

When enzyme activity was converted to a per leaf basis, there was no difference between the treated and untreated preparations on the first and fourth days (Figure 34). The treated preparations exhibited a sharp decrease in enzyme activity by the seventh day.

Decreased enzymatic activity was found for both treatments on the seventh day with data based on fresh weight; however, the decrease was more pronounced for treated preparations (Figure 35). The decrease for the untreated preparations can be attributed to a

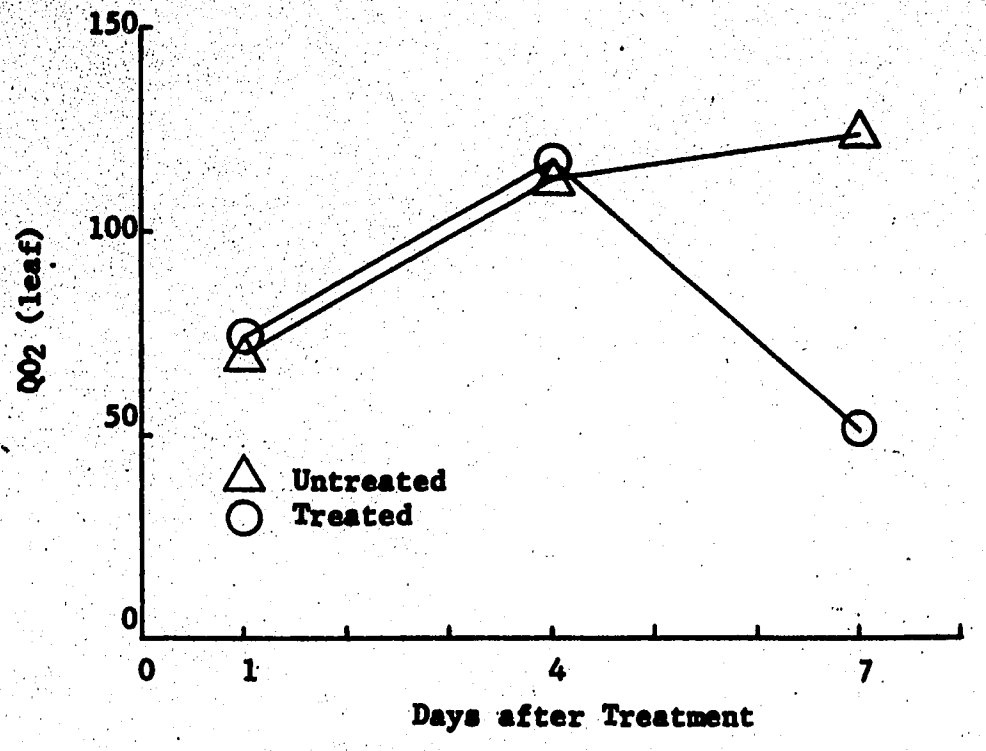


Figure 34. The effect of DIURON-DBSA on the oxidation of glycolic acid by soybean primary leaves.

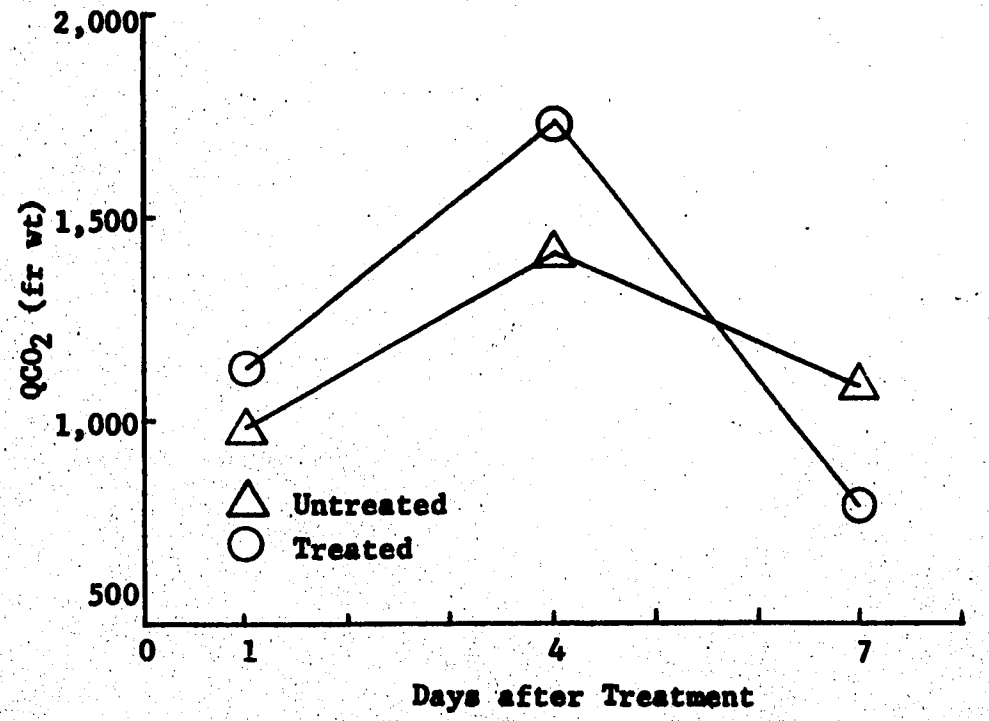


Figure 35. The effect of DIURON-DBSA on the glycolic acid oxidase activity per gram of leaf tissue.

more rapid increase in fresh weight than increase in enzymatic activity. The decrease for the treated preparations is mostly due to a decrease in enzyme activity as treated leaves show smaller increases in weight during this period. Increased activity of the treated over the untreated preparations is notable on the fourth day. This was usually observed with the raw data (μL O_2/2ml supernatant) or when the data were expressed on a fresh weight basis. Funderburk and Porter (1961) reported that DCMA reduced glycolic acid oxidase from corn leaves. However, Bingham and Porter (1961c) reported that DCMA had no effect on glycolic acid oxidase activity when based on nitrogen.

In other experiments, the cell wall fraction from simple leaves was found to oxidize glycolic acid. The second supernatant (10,000 X G) from the washing of "particles" likewise oxidized glycolic acid. Considerable less activity was found in the third supernatant.

Sodium bisulfite was initially tried as an inhibitor of glycolic acid oxidase at a concentration of one mM (Figure 36). An increase in enzyme activity was found at this concentration. This result was contrary to that reported by other investigators (Bingham and Porter, 1961c; and Funderburk and Porter, 1961). Other inhibitors were also studied (Figures 37 and 38).

Two-tenths mM DDC had no effect on the glycolic acid oxidase activity of untreated or treated preparations. One mM KCN and acetaldehyde bisulfite appeared to have an inhibitory effect on the enzymatic activity of untreated plants. The effect of cyanide

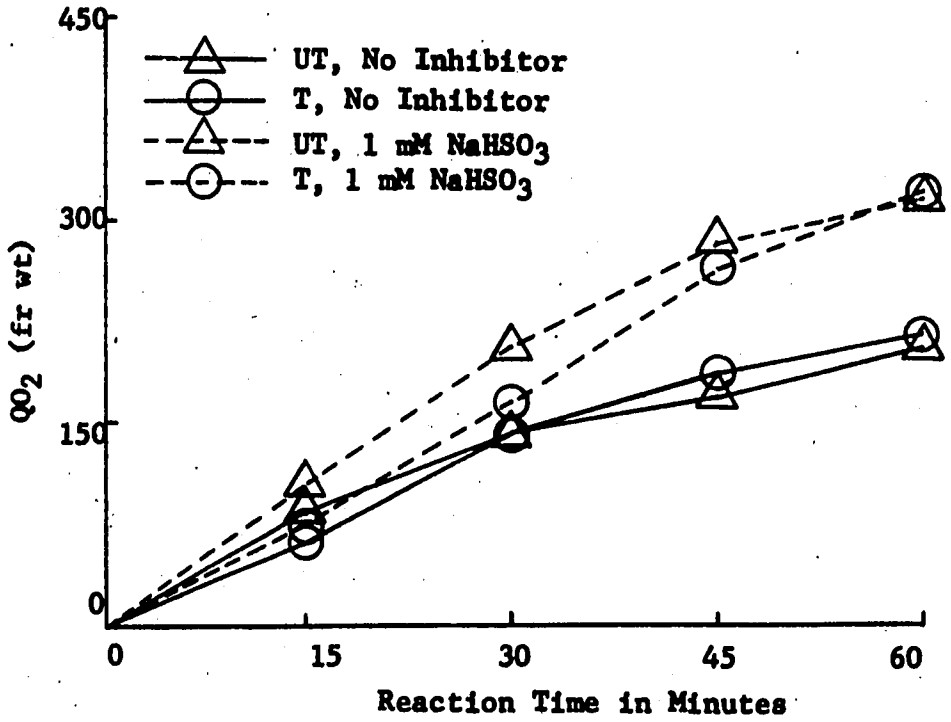


Figure 36. The effect of sodium bisulfite on glycolic acid oxidase activity seven days after DIURON-DBSA treatment.

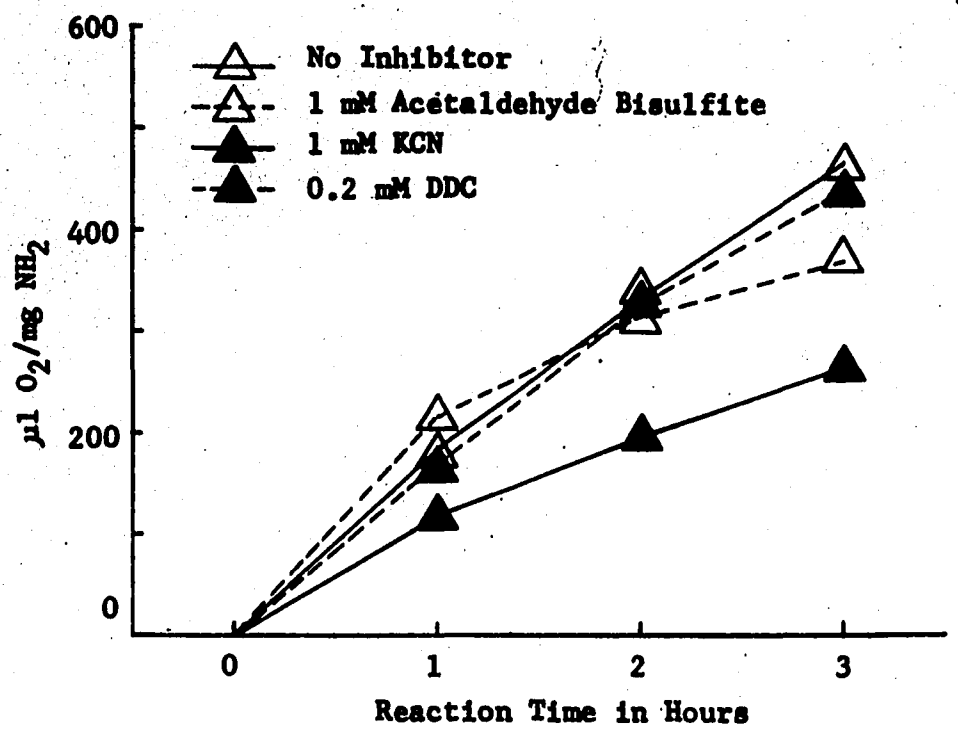


Figure 37. The effect of various inhibitors on the glycolic acid oxidase activity of 1,000 X G supernatants from 12 day old untreated primary leaves.

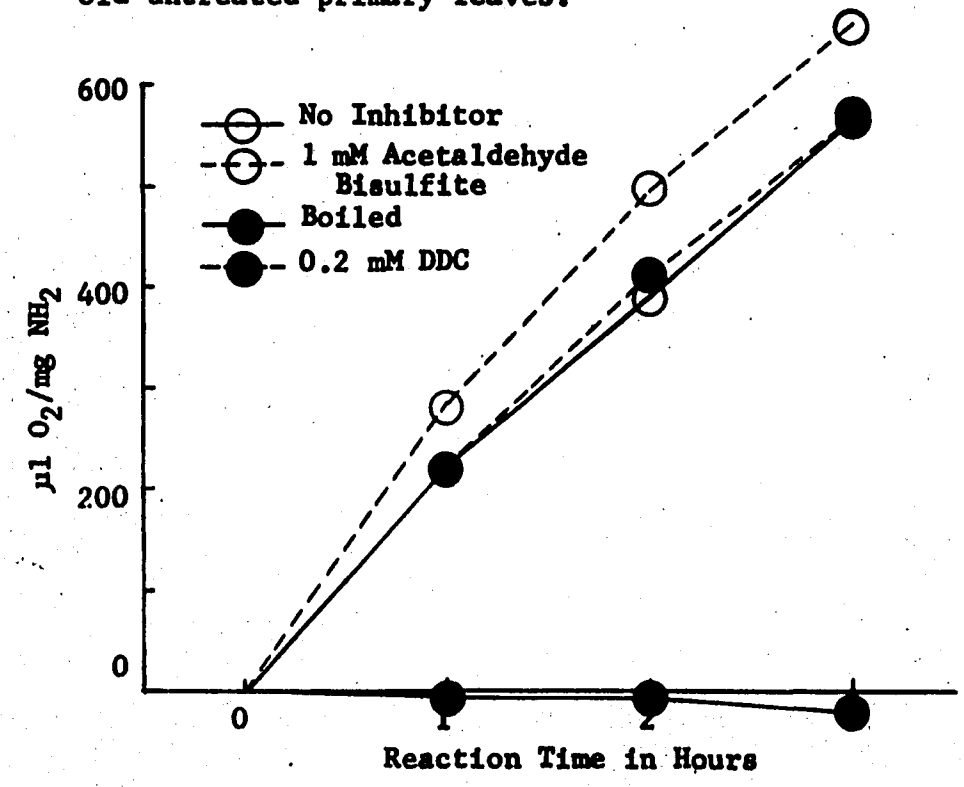


Figure 38. The effect of various inhibitors on the glycolic acid oxidase activity of 1,000 X G supernatants from 12 day old treated primary leaves.

is suprising because oxygen uptake should be increased through the inhibition of catalase. With the treated preparations, one mM acetaldehyde bisulfite appeared to stimulate enzyme activity (Figure 38). Boiling destroyed the oxidation of glycolic acid, which indicates that the oxidation was enzymatic in nature. It was also notable that the treated preparation was more active than the untreated. This was commonly observed on the fourth day after herbicide treatment.

The failure to get effective inhibition of glycolic acid oxidase with the bisulfites was further investigated. A logarithmic series of concentrations of acetaldehyde bisulfite was used. One-tenth mM had little effect or was stimulatory (Figure 39). One mM and 10 mM concentrations gave some inhibition, although 10 mM was not as effective as Bingham and Porter (1961c) reported for one mM. The stimulation of enzyme activity by low concentrations of an inhibitor is usually attributed to the elimination of competing reactions (Hackett, 1960).

One of the products of glycolic acid oxidase activity is hydrogen peroxide which is in turn broken down to water and oxygen by the enzyme catalase. Since catalase is inhibited by cyanide and glycolic acid oxidase is not, the effect of cyanide inhibition of this coupled reaction should be a greater net oxygen consumption. This was investigated by means of a logarithmic series of concentrations of cyanide (Figure 40). A stimulation of glycolic acid oxidase activity was found for a cyanide concentration of 10 mM.

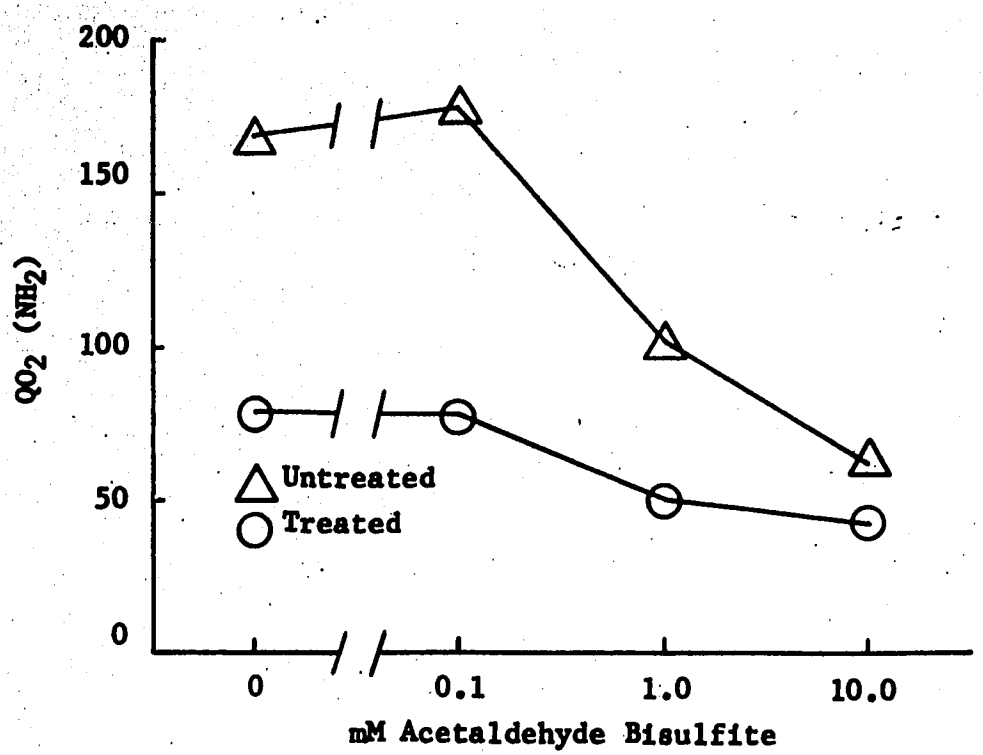


Figure 39. The effect of acetaldehyde bisulfite on glycolic acid oxidase activity seven days after herbicide treatment.

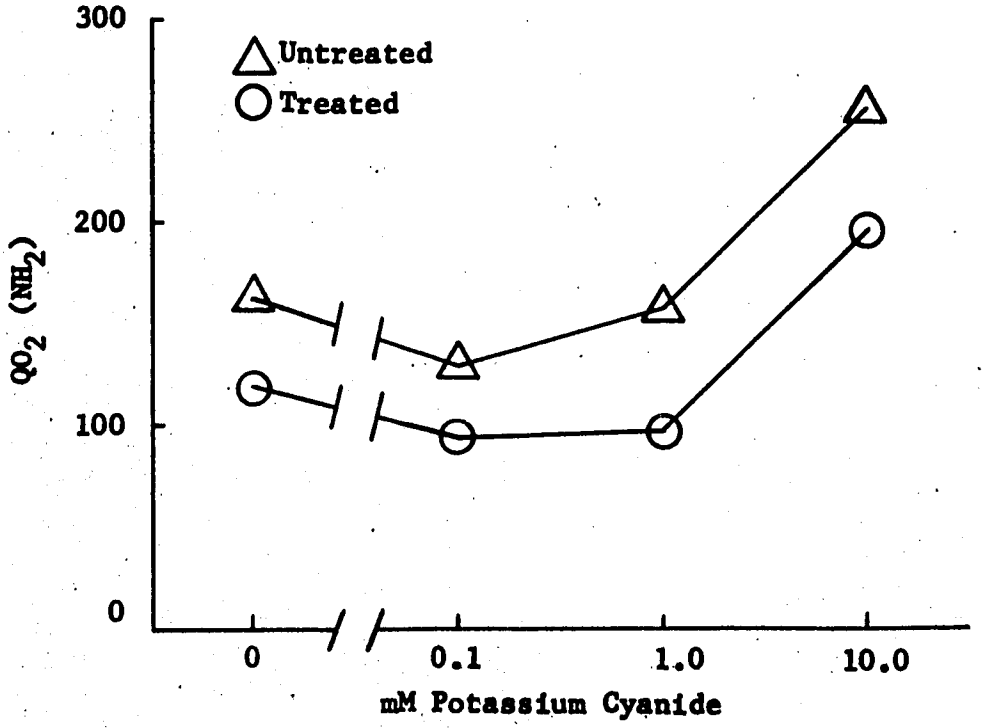


Figure 40. The effect of potassium cyanide on glycolic acid oxidase activity four days after herbicide treatment.

Bingham and Porter (1961c) reported the same result. The reduction in oxygen uptake at 0.1 and 1.0 mM concentrations could possibly be attributed to a stimulation in catalase activity with the production of increased amounts of oxygen. Potassium cyanide had been found to be a very effective inhibitor of catalase at a concentration of one mM. In that case, however, the enzyme preparation had been diluted ten-fold. This may possibly be the reason for the failure to find inhibition at a one mM concentration.

Glycolic acid oxidase activity was high and the enzyme appeared to have typical properties except for a lack of sensitivity to sodium and acetaldehyde bisulfite. No explanation appears evident for this result.

SUMMARY

Various growth, anatomical, and physiological effects of DIURON-DBSA treatment on soybeans were studied.

Injury to leaves due to foliar application was restricted to the site of application; however, the herbicide was found to be absorbed from nutrient solutions and gave top kill at concentrations greater than 0.1 ppm. Spray application of the herbicide at the rate of three-eighths pound per acre produced mottled chlorosis by the fourth day after herbicide treatment. Height growth of plants was inhibited by the herbicide treatment. The greatest effect was on the internode between the primary leaves and the first trifoliate leaf. Treated primary leaves showed less increase in fresh weight than the control treatment.

Total amino nitrogen contents of primary leaves were unaffected by the herbicide treatment. The proportion of non-protein amino nitrogen was increased 50 per cent by the herbicide treatment. Nitrogen concentration decreased on a fresh weight basis during the testing period. The decrease was less for treated primary leaves because of smaller gains in fresh weight.

Microscopic examination of anatomical slides indicated that the roots and the internode between the cotyledons and primary leaves were unaffected by the herbicide treatment. The internode between the primary leaves and the first trifoliate leaf was not studied. Study of cross-sections of the primary leaves indicated

that cell sizes were generally reduced by the herbicide treatment. Enlargement and differentiation of xylem vessels were particularly inhibited.

A two-day dark period prior to the herbicide treatment reduced the time period required for the appearance of injury symptoms. With light treatment before and after the herbicide treatment, the plants tended to recover from the herbicide when applied at the rate of one-fifth pound per acre.

The effect of the herbicide treatment was to reduce oxygen consumption and carbon dioxide evolution by leaf disks from primary leaves. With light treatment before and after herbicide treatment, the respiratory activities tended to recover from the herbicide treatment. Respiratory activities continued to decline with a dark after treatment. The RQ values increased very markedly with the combination of dark after-treatment and herbicide treatment.

Cytochrome oxidase activity could not be demonstrated in particles from green stems. Particles were isolated from etiolated cotyledons that oxidized succinate, pyruvate, and malate. The oxidation of succinate was found to be reduced slightly by herbicide treatment.

A very active enzymatic oxidation of ascorbic acid was found. The enzymatic activity was localized in the cell walls of primary leaf homogenates. Diethyldithiocarbamate and 8-hydroxyquinoline were effective inhibitors of ascorbate activity. Leaf preparations were more active than homogenates from cotyledons. The herbicide treatment did not have any noticeable effect on the oxidation of ascorbic acid.

Polyphenol oxidase activity was very low in soybean primary leaves, although activity tended to increase with leaf age. The cotyledons exhibited no polyphenol oxidase activity. Diethyldithiocarbamate and 8-hydroxyquinoline were found to inhibit polyphenol oxidase activity. The enzyme activity of preparations from treated leaves remained constant from the fourth to the seventh days while that of the untreated preparations increased in activity.

Catalase activity of primary leaf preparations was high and activity increased with leaf age. One mM potassium cyanide gave nearly complete inhibition of catalase activity. Catalase activity was reduced approximately one-half by the herbicide treatment, while the enzyme activity of check plants increased with leaf age.

Peroxidase activity was low, although activity increased with leaf age. Cyanide inhibited peroxidase activity at one mM concentration. The herbicide treatment reduced peroxidase activity on the fourth and seventh days after the herbicide treatment similarly to the reduction in catalase activity.

Glycolic acid oxidase activity of primary leaf preparations was high and compared well with ascorbic acid oxidase activity. Acetaldehyde bisulfite and sodium bisulfite were found to be poor inhibitors of glycolic acid oxidase activity. Net oxygen uptake could be increased with 10 mM potassium cyanide, which indicated that the catalytic breakdown of hydrogen peroxide by catalase was inhibited.

Glycolic acid oxidase activity increased with leaf age on a nitrogen basis. On a fresh weight basis, enzyme activity

increased to the fourth day and sharply decreased on the seventh day. When enzyme activity was calculated on a per leaf basis, enzyme activity of the untreated preparations continued to increase throughout the testing period. Enzymatic activity decreased sharply from the fourth to the seventh day with the herbicide treatment.

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BIOGRAPHY

Arnold David Lewis was born near Rose Hill, Mississippi, on September 8, 1931. He was graduated from Rose Hill Consolidated High School and entered Jones County Junior College in 1949. He was called to active military service in August, 1950, and served until March, 1952, in the Army Medical Service Corps. He returned to Jones County Junior College and received the degree of Associate in Arts in August, 1952.

He enrolled at Louisiana State University in September, 1952, and received the degree of Bachelor of Science in Forestry in June, 1955. He was employed by the Mississippi Forestry Commission for one-half year before being employed by the Forestry department of the Mississippi Agricultural Experiment Station.

He enrolled in Graduate School at Auburn University in September, 1957, and remained there until September, 1958. He was employed by the United States Forest Service until December, 1959. He then returned to Auburn University and completed the requirements for the degree of Master of Science in Forestry. He enrolled in June, 1960, at Louisiana State University as a Graduate Research Assistant in the Department of Botany and Plant Pathology. He is a candidate for the degree of Doctor of Philosophy in January, 1963.

EXAMINATION AND THESIS REPORT

Candidate: Arnold David Lewis

Major Field: Botany

Title of Thesis: SOME EFFECTS OF DIURON-DBSA ON EARLY GROWTH, DEVELOPMENT AND CERTAIN RESPIRATORY ENZYMES OF SOYBEAN

Approved:

John B. Baker
Major Professor and Chairman

Max Goodrich
Dean of the Graduate School

EXAMINING COMMITTEE:

Leon C. Standifer

Lewis H. Flint

M.B. Sturgis

St. John P. Chilton

W.C. Norman

Date of Examination:

January 8, 1963